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Award Number: DAMD17-00-1-0492

TITLE: Ex Vivo Expansion of HER2-Specific T Cells for the
Treatment of HER2-Overexpressing Breast Cancer

PRINCIPAL INVESTIGATOR: Keith L. Knutson, Ph.D.

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA 98105-6613

REPORT DATE: May 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE May 2002	3. REPORT TYPE AND DATES COVERED Annual Summary (3 Apr 01 - 2 Apr 02)		
4. TITLE AND SUBTITLE Ex Vivo Expansion of HER2-Specific T Cells for the Treatment of HER2-Overexpressing Breast Cancer		5. FUNDING NUMBERS DAMD17-00-1-0492		
6. AUTHOR(S) Keith L. Knutson, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Washington Seattle, WA 98105-6613 E-Mail: kknutson@u.washington.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (<i>Maximum 200 Words</i>) Adoptive T cell therapy has the potential to eradicate existing malignancy in humans. I have been investigating the immune effector arms most efficacious in eradicating malignancy in the neu-transgenic mouse model. These mice develop spontaneous tumors that are histologically similar to those observed in humans. Two helper peptides, p781-795 and p1166-1180, of neu have been identified to which T helper cell lines can be generated. The T cell lines are CD4+ and demonstrate a Th1 phenotype with the production of IFN-γ but not IL-4. Cell lines derived using these peptides, were tested for the ability to eradicate existing bulky malignancy. It was observed that T cell injection resulted in a partial tumor response when cells line were used individually. Small increases in survival time were observed. When the cell lines were combined, survival was also improved and some tumors had lost antigen neu expression at the cell surface. In addition to Th1 immunity it is hypothesized that other immune effector arms, such as Th2, could also be effective. Vaccination strategies have been developed that elicit Th2 T cells. Expansion of Th2 cells ex vivo and their therapeutic efficacy is being examined. A monoclonal antibody therapy strategy has also been developed that will be tested in combination with adoptive T cell transfer. Techniques for optimal ex vivo expansion of human HER-2/neu-specific T cells are also being developed. Peptide presentation during culture can impact antigen-specific T cell responsiveness of human T cell lines. The inclusion of IL-12 during culture with HER-2/neu peptides can greatly enhance the antigen-specificity of the cultures. The findings in the animal model and ex vivo expansion of human T cells will be directly translated to human clinical trials of adoptive T cell therapy.				
14. SUBJECT TERMS breast cancer, adoptive immunotherapy, T cells, immunology, peptide-based vaccine, cytolytic T cell, T helper cell, cytokines			15. NUMBER OF PAGES 117	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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Appendix 1: Expansion of HER-2/neu specific T cells *ex vivo* following immunization with a HER-2/neu peptide based vaccine.

Appendix 2: Diversity of the T cell population responding to a dominant HLA-A2 epitope of HER-2/neu in an ovarian cancer patient.

Appendix 3: IL-12 enhances the function and number of tumor antigen specific Th1 lymphocytes during *ex vivo* expansion.

Appendix 4: Adoptive T cell therapy for the treatment of solid tumors.

Appendix 5: Immunotherapy of Breast Cancer.

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Appendix 7: An MHC Class II epitope derived from the neu oncoprotein will elicit differential levels of immunity after immunization in parental vs. mice transgenic for neu.

Appendix 8: Vaccination against the HER-2/neu oncogenic protein.

Introduction:

A central hypothesis of this grant proposal is that tumors can be eradicated *in vivo* using adoptive transfer of tumor-specific T cells. However, many of the underlying principles of adoptive T cell therapy are not well understood. This grant describes experiments that aim to elucidate some of these principles. The specific aims of this proposal are to, (1) determine the immune effector arm most effective in the eradication of rat neu overexpressing tumors in neu-tg mice using adoptive immunotherapy of rat neu specific CD8+ and CD4+ T cells and (2) determine the feasibility of the expansion of HER2 specific CD4+ and CD8+ T cell lines from patients previously immunized with a HER2 peptide-based vaccine.

Body:

1. To determine the immune effector arm most effective in the eradication of rat neu-overexpressing tumors in neu-tg mice using adoptive immunotherapy. Work in year two of the proposal has consisted of generating T cells, characterizing the T cells *ex vivo*, and infusing them into animals to assess an anti-tumor response. Mice were immunized with peptides derived from the neu protein sequence that had been shown to be immunogenic and result in a protein, thus potentially, a tumor specific response (Appendix 1). Spleens from immunized animals were removed and peptide-specific T cells expanded *ex vivo* using IL-2. The first peptide, p781-795, could generate detectable peptide specific T cells directly from the spleen after immunization (Fig. 1A) which could be highly enriched after 2 *in vitro* stimulations (IVS) (Fig. 1 B). The lines generated using this 15-mer peptide were >95% CD4+. In addition, the peptide-specific T cell line could recognize neu overexpressing tumor cells derived from the neu transgenic mouse and specifically secreted IFN γ (Fig. 1B). When the p781-specific T cell lines were infused into tumor bearing animals tumor growth was inhibited, but not completely

suppressed (Fig. 2A) and overall survival of the animals was prolonged (Fig. 2B). Similarly, T cell lines could be generated with a second peptide, p1166-1180, another 15-mer derived from the rat neu protein sequence. The peptide-specific T cells generated after 2 IVS were strongly Th1, IFN γ producing, and could respond to endogenous tumor, albeit at a much lower level than peptide (Fig. 3). The p1166-specific T cells, when infused into tumor bearing mice, could substantially inhibit tumor growth (Fig. 4A). Survival of the mice was only minimally prolonged (Fig. 4B). When the two lines were combined to generate a polyclonal antigen specific Th1 CD4 $^{+}$ line and infused into tumor bearing animals a third of the mice had prolonged survival (Fig. 5). Thus, the generation of T cell lines specific for both peptide and tumor cells is possible after active peptide immunization of the neu transgenic mice. In addition, the phenotype of these lines is CD4 $^{+}$ Th1. Infusion of these lines singly results in an anti-tumor response and the anti-tumor response can be increased when the lines are infused together in a polyclonal fashion. In some animals that had received polyclonal T cell infusions, neu expression was down regulated indicating the potential for the generation of an antigen-negative variant (Fig. 6).

A unique aspect of the HER2 protein is that a Th2 response may also be beneficial in the augmentation of HER2 specific antibodies that may have an effect against the neu receptor. I immunized animals with a protein-based vaccine and were able to generate both neu-specific T cells (Fig. 7A) and high levels of neu specific antibodies (Fig. 7B). Furthermore the T cells were characterized to be of a Th2 phenotype (Fig. 8), secreting IL-4 (Fig. 8A) but not IFN- γ (Fig. 8B). Studies are ongoing evaluating the infusion of Th2 neu-specific T cells for their ability to inhibit tumor growth.

I have also begun to evaluate the efficacy of combining adoptive T cell therapy with neu-specific monoclonal antibody therapy. In humans, HER-2/neu-specific monoclonal antibody therapy is already known to reduce tumor growth rates. An antibody, 7.16.4, which has been previously examined for inhibition of tumor growth, is available. Monoclonal antibody therapy was examined in animals with established bulky malignancy. Tumor-bearing animals were infused by tail vein injection with 10 μ g of 7.16.4 either on day 10 or day 14 following tumor challenge. Control animals were treated with PBS. Tumor growth was monitored every 2 days revealing that 7.16.4 therapy resulted in significantly reduced tumor growth rates (Fig. 9A). Monoclonal antibody therapy targeting neu can only be combined with adoptive T cell therapy if the antibody therapy does not downregulate cellular expression of neu. Thus we investigated the mechanism of action of antibody therapy with *in vivo* monitoring using radiolabeled probes and flow cytometric analysis (Fig. 9B-C). Antibody therapy significantly reduced both the metabolism and proliferation of the tumor cells (Fig. 9B). Importantly, antibody therapy does not result in decreased levels of neu expression (Fig. 9C). These results indicate that antibody therapy targeting neu could potentially be used in combination with adoptive T cell therapy. These studies are currently underway.

Finally, we have begun to evaluate the efficacy of tumor-specific T cells generated from animal where the antigen is a "self" protein as compared to animals where the antigen is "foreign" utilizing the neu transgenic mouse and the parental strain from which the transgenic was derived the FVB/N mouse. Tumors grow readily in the neu transgenic mouse at a dose of 5×10^5 (Fig. 10) but are rejected after implantation in the syngeneic parental FVB/N where the neu antigen is a foreign protein (Fig. 10B). In addition, spleens removed after tumor implant show no evidence of a T cell response directed against neu tumor in the transgenic animal (Fig. 10C) as compared to a significant T cell response against the neu tumor in the FVB/N parental strain where tumor had been rejected (Fig. 10C). T cells from both animals are responsive to mitogenic stimulation as assessed by exposure Concanavalin A-elicited blasts. When the parental animal is given a large bolus of tumor (10^7 cells), after an initial tumor rejection the tumors will grow *in vivo*. The growth rate of the neu-expressing tumors is substantially slower than the growth of tumor in the transgenic animals. When tumors are harvested from the parental animals after 40 days *in vivo* the masses are infiltrated with T cells (Fig. 11C). Furthermore, the tumors have lost nearly all the cells expressing the neu antigen as compared to tumor cells from neu-transgenic mice (Fig. 11A-B) indicating effective immunity generating antigen negative variants.

2. To determine the feasibility of the expansion of HER2-specific CD4 $^{+}$ and CD8 $^{+}$ T cell lines from patients previously immunized with a HER2 peptide-based vaccine. Competition among peptides for MHC class I and class II binding may diminish antigen-specific responsiveness following *ex vivo* expansion. Cultures carried through *in vitro* stimulation (IVS) alone in neu peptide p776 had a greater response to peptide antigen than

cultures carried through IVS in a mixture of peptides that included p776 (Fig. 12). Similar findings have been observed for other peptides. Thus, *ex vivo* expansion may be best achieved when peptide immunogens are kept separate. Studies are continuing to examine the most effective mode of antigen presentation.

With respect to cytokines, studies to date have evaluated IL-2+ IL-7 (Year 1 annual report), IL-2+ IL-15 (Year 1 annual report), and IL-2+ IL-12 as cytokine growth factors for the *ex vivo* expansion of human HER2 specific T cells from the PBMC of cancer patients after active immunization. For the current year I have been further evaluating IL-12 which has led to submission of a manuscript assessing the utility of IL-12 markedly enhances both the number, as well as function, of Th1 T cells generated in culture (Figs. 13-14). Data from 2 patients is shown. Cultures generated with limited IVS, in patients who have developed specificities to multiple peptides after immunization demonstrate that polyclonality can be preserved (Fig. 15). Patient 5922 developed immune responses to two peptides in her immunizing mix, p369 and p927 as well as epitope spreading to peptides p971 and p776. She did not respond to one peptide in her immunizing mix, p688, after active immunization. Bulk cultures established in this patient and taken through 3IVS with peptide p971 along with IL-2 and IL-12 resulted in a polyclonal line that had enhanced specificity and number of all responding peptide specific cells. There was no effect in the number of cells to the peptide to which she had not developed the immune response. Studies are ongoing to determine whether specificity and polyclonality will be preserved after non-specific expansion. Furthermore, studies with CD40L are currently being planned (See conclusions for future directions).

Key Research Accomplishments:

- Th1 T cell lines specific for neu oncoprotein can be elicited from animals following active immunization.
- Th1 T cell lines specific for neu oncoprotein are partially effective at inhibiting tumor growth in neu-transgenic mice.
- Injections of combinations of T cells lines can improve survival to a greater extent than that of lines specific for a single peptide.
- Antigen-negative variants can be elicited as a result of a neu-specific immune response.
- Th2 T cells can be generated by active immunization with the neu protein.
- Monoclonal antibody therapy greatly suppresses tumor growth without the loss of neu antigen expression suggesting potential for combination therapy with adoptive T cell transfer.
- Expansion of human HER-2/neu-specific T cells *ex vivo* with single peptides is better than using a mixture of peptides.
- IL-12 is effective at enhancing the numbers of antigen-specific Th1 T cells when combined with IL-2
- IL-12 is effective at enhancing the function of antigen-specific Th1 cells when combined with IL-2.

Reportable outcomes:

(Published or submitted pertaining to the application during the funding period (April, 2001-April, 2002))

1. **Knutson, K.L.** and Disis, M.L.: Expansion of HER-2/neu specific T cells *ex vivo* following immunization with a HER-2/neu peptide based vaccine., Clin. Breast Cancer. 2:73-79, 2001.
2. **Knutson, K.L.** and Disis, M.L.: Diversity of the T cell population responding to a dominant HLA-A2 epitope of HER-2/neu in an ovarian cancer patient. (in press) Human Immunology, 2002.
3. **Knutson, K.L.** and Disis, M.L.: IL-12 enhances the function and number of tumor antigen specific Th1 lymphocytes during *ex vivo* expansion. Submitted, 2002.
4. **Knutson, K.L.** Almand, B., Mankoff, D.A., Schiffman, K., and Disis, M.L.: Adoptive T cell therapy for the treatment of solid tumors. Expert Opinion in Biological Therapy, 2:55-66, 2002.
5. **Knutson, K.L.**, Bishop, M.R., Schiffman, K., and Disis, M.L.: Immunotherapy of Breast Cancer. Cancer Chemotherapy and Biologic Response Modifiers- Annual 20. Editors: Giaccone, Schilsky and Sondel; Elsevier Science (in press), 2001.

6. **Knutson, K.L.**, Ahlquist, M., and Disis, M.L.: Immunization with the intracellular domain (ICD) protein of HER-2/neu (HER2) generates predominantly Th2 immunity in neu-transgenic mice. Proc AACR, 43:605, 2002 (abstract).
7. **Knutson, K.L.**, Ahlquist, M., and Disis, M.L.: An MHC Class II epitope derived from the neu oncoprotein will elicit differential levels of immunity after immunization in parental vs. mice transgenic for neu. Proc. AACR, 43:1111-1112, 2002 (abstract).
8. Bernhard, H., Salazar, L., Schiffman, K., Smorlesi, A., Schmidt, B., **Knutson, K.L.**, Disis, M.L.: Vaccination against the HER-2/neu oncogenic protein. Endocrine Related Cancer, 9:33-44, 2002.
9. Disis, M.L., Rinn, K., **Knutson, K.L.**, Davis, D., Caron, D., dela Rosa, C., Schiffman, K.: Flt3 ligand as a vaccine adjuvant in association with HER-2/neu peptide-based vaccines in patients with HER-2/neu-overexpressing cancers. Blood, 99:2845-2850.
10. Disis, M.L., Gooley, T.A., Rinn, K., Davis, D., Piepkorn, M., Cheever, M.A., **Knutson, K.L.**, and Schiffman, K.: Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. Journal of Clinical Oncology, (in press).

Conclusions

I have demonstrated that neu-specific T cells can be expanded *ex vivo* from neu transgenic mice that have been immunized with peptide based vaccines and that Th1 CD4+ T cells can mediate an anti-tumor effect *in vivo*. This is the identical model that our group has established to test in the human system using previously vaccinated patients with HER2 overexpressing breast and ovarian cancers. In addition, I have determined that Th2 CD4+ T cells can be generated and isolated from the neu transgenic mouse. However, work performed in the funding period also demonstrated that one of the potential pitfalls of adoptive immunotherapy may be the generation of antigen negative variants. By reducing the aggressiveness of tumor growth rates using monoclonal antibody therapy combined with adoptive T cell therapy, I may be able to demonstrate tumor regression without the generation of antigen-negative variants. Human HER2-specific T cells can be best expanded *in vitro* using single peptide cultures, rather than combining the peptides. Finally, human tumor specific T cells can be expanded culture using IL-2 and IL-12 and polyclonality as well as epitope spreading present in these T cell populations is preserved with *ex vivo* expansion. My future plans for each of the specific aims are:

1. To determine the immune effector arm most effective in the eradication of rat neu-overexpressing tumors in neu-tg mice using adoptive immunotherapy of rat neu specific CD8+ and CD4+ T cells. Work will focus on the arm of the immune system involved in tumor eradication both via cell population depletion studies as well as directed infusion of neu specific Th1, Th2 CD4+ T cells as well as CD8+ CTL generated by stimulation with syngeneic tumor. Neu specific antibody can be generated and Ig will be purified to determine the role of IgG in potentially enhancing the efficacy of the neu specific T cell response. Alternatively, therapy with a purified "trastuzumab-like" antibody during T cell transfer may also promote regression. Tumors will be evaluated for mechanisms of failure of adoptive immunotherapy such as lack of T cell homing or the generation of antigen negative variants when treatment failures occur. These findings may be directly translational to the clinical setting of HER-2/neu-overexpressing breast cancer.

2. To determine the feasibility of the expansion of HER2 specific CD4+ and CD8+ T cell lines from patients previously immunized with a HER2 peptide based vaccine. I will further investigate expansion with CD40L which has been obtained from Immunex Corporation (Seattle, WA) specifically for these studies. In an agreement with Xcyte Technologies (Seattle, WA), I will obtain beads coated with anti-CD3 and anti-CD28 antibodies, which have been shown to activate T cells. I will begin to develop clinical SOPs for the expansion of tumor-specific T cells based on our work with IL-2 and IL-12 with an expansion step using GMP produced anti-CD3/anti-CD28 beads. This data will be used this to submit an IND for a clinical trial in the last quarter of 2002.

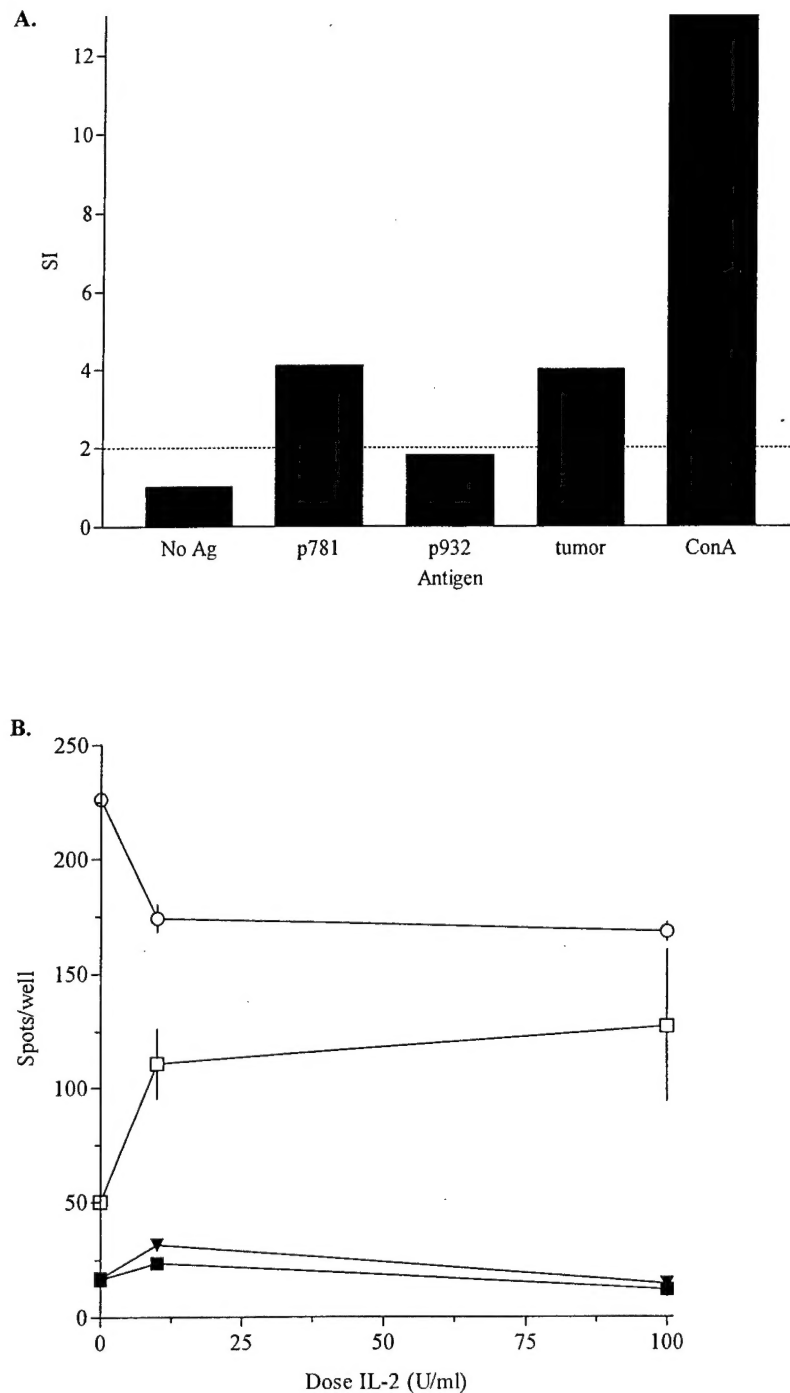


Figure 1. T cells specific for rat neu peptide p781 can be elicited by active immunization and expanded *ex vivo*. A. Mice immunized twice with 100 μ g p781 have detectable T cell response (S.I. >2) to the immunizing peptide and rat neu overexpressing tumor cells, but not to an irrelevant peptide, p932. The data is expressed as a stimulation index on 6 well replicates. Values above a stimulation index of 2 (dotted line) are statistically significant ($p < 0.05$). B. T cells can be expanded on 2 IVS with peptide, p781 and 5 U/ml IL-2. Open circles represent IFN γ releasing cells responding to p781 and open squares are the peptide specific cells responding to tumor. Closed triangles are no antigen and closed squares represent response to irrelevant peptide p932. Each data point represents duplicate determinations.

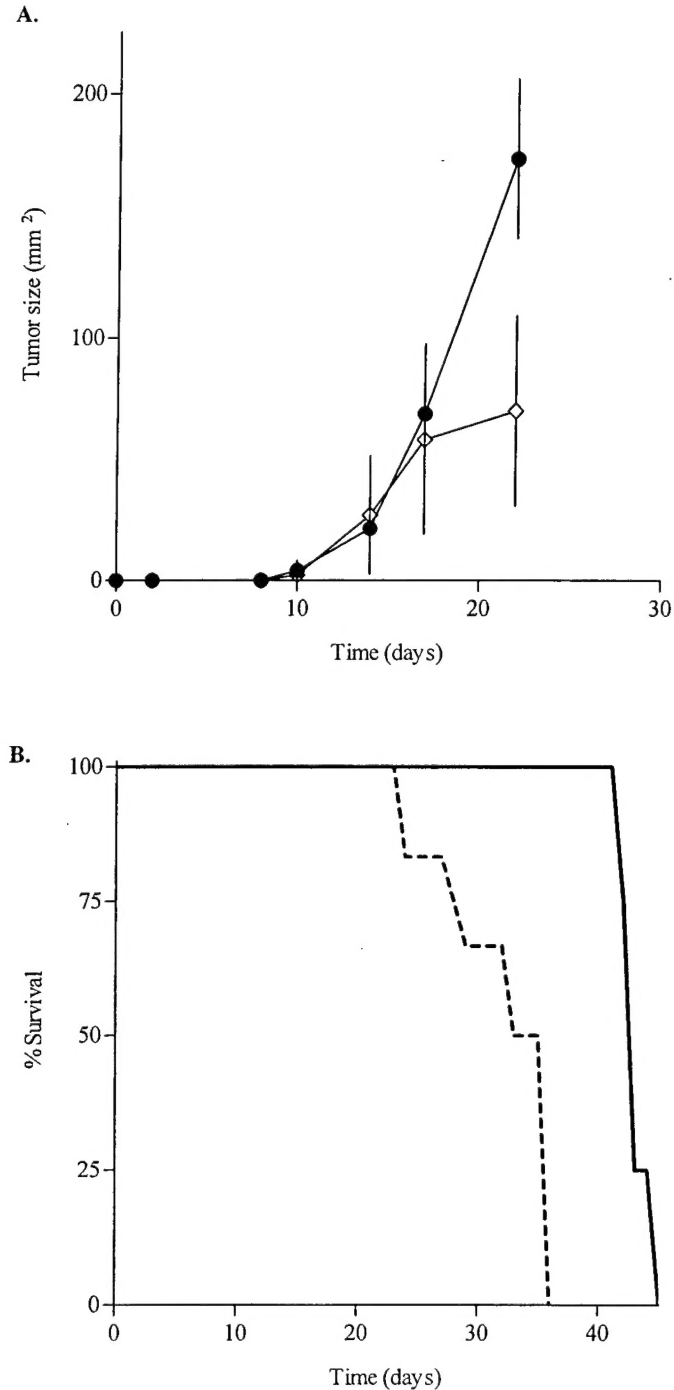


Figure 2. p781 specific T cells can mediate an anti-tumor response. A. 5×10^6 p781 specific T cells were infused into animals 24 hours after a 5×10^6 tumor cell implant could inhibit tumor growth. Data shown as the mean and standard deviation of 5 mice/group, those that received p781 specific T cells (open diamonds) or PBS (closed circles). At 22 days following tumor injection, the mean tumor size of the treated group was significantly less than the control group. B. p781-specific T cell lines (characterized in Fig. 1) were infused into animals at a dose of 5×10^6 cells on the same day (0) that 5×10^6 syngeneic tumor cells were administered sq. Animals receiving T cell infusions (bold line) survived longer than animals receiving PBS (dotted line).

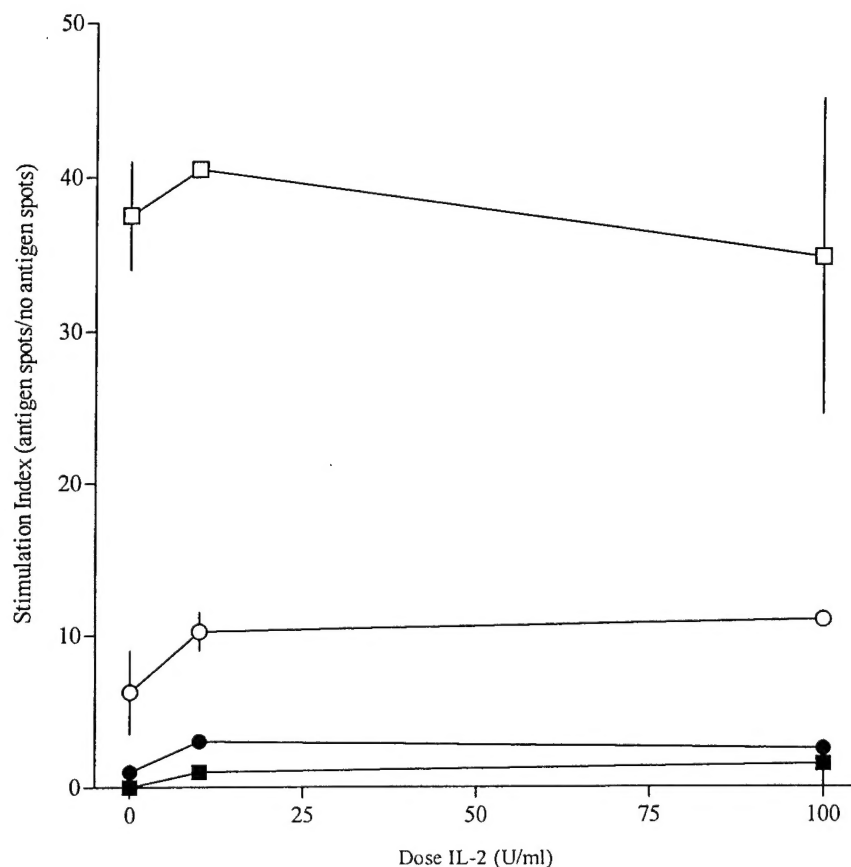


Figure 3. T cells specific for rat neu peptide p1166 can be elicited by active immunization and expanded *ex vivo*. After 2 *in vivo* peptide immunizations p1166 specific T cells can be detected and expanded *ex vivo* with peptide and IL-2. After 2 IVS the T cell line is both p1166 specific (open squares) and tumor specific (open circles). The line is IFN γ producing and does not respond to no antigen (closed circles) or an irrelevant peptide (closed squares). Each symbol is the mean of duplicate determinations.

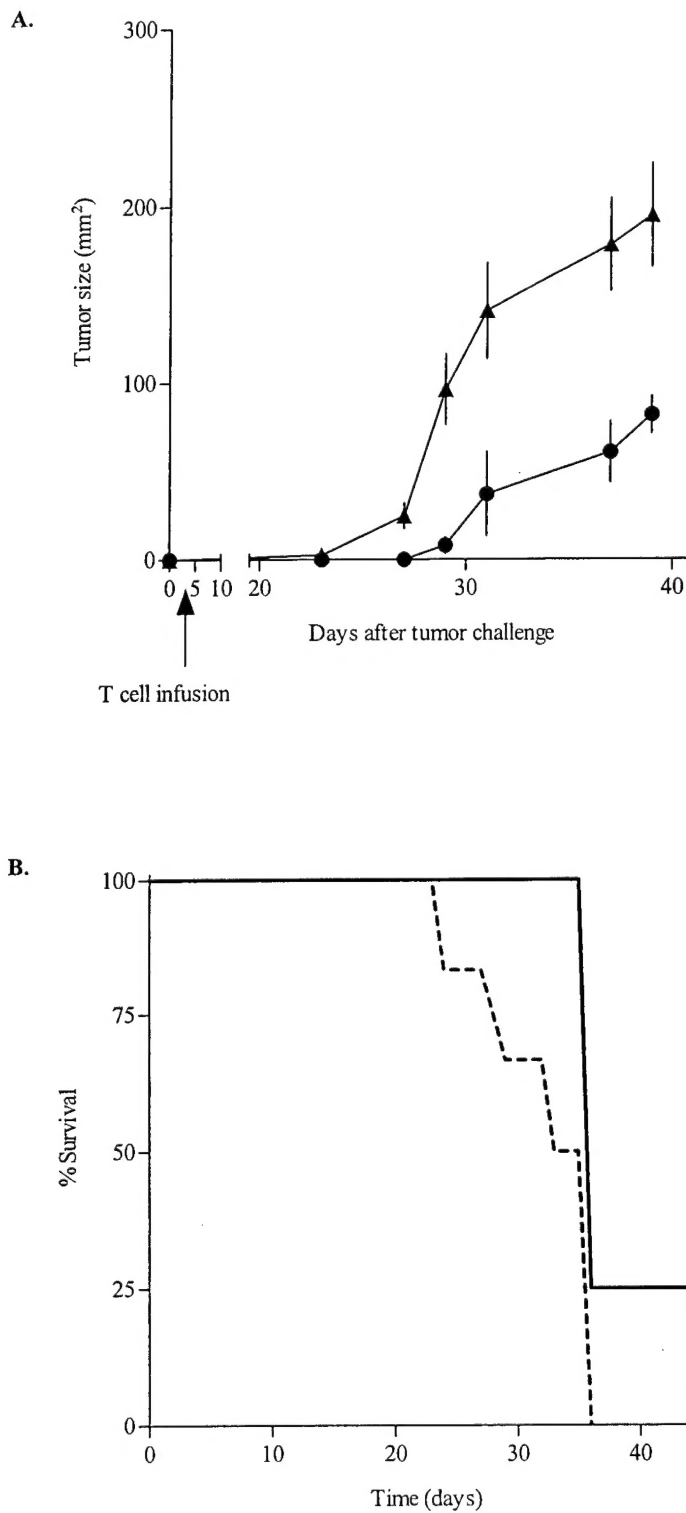


Figure 4. p1166 specific T cells can mediate an anti-tumor response.
 A. 5×10^6 p1166 specific T cells were infused into animals 24 hours after a 5×10^6 tumor cell implant was placed. p1166 specific T cells could inhibit tumor growth (closed circles) compared to PBS (closed triangles). B. Animals infused with p1166 specific T cells (bold line) had a survival benefit compared to animals infused with PBS (dotted line). Data represents 5 animals/group.

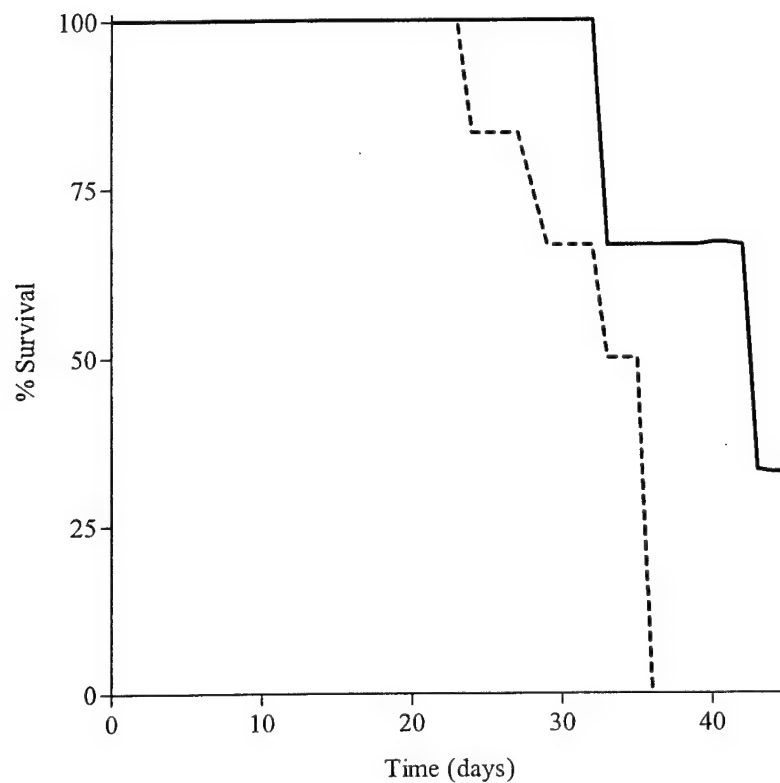


Figure 5. Polyclonal T cell infusions have improved therapeutic efficacy compared to infusion of T cells for a single specificity. Animals were infused with a combination of p781 and p1166 specific T cell lines (bold line) and compared to PBS alone (dotted line). Polyclonal T cell infusions resulted in 40% of mice having improved long term survival.

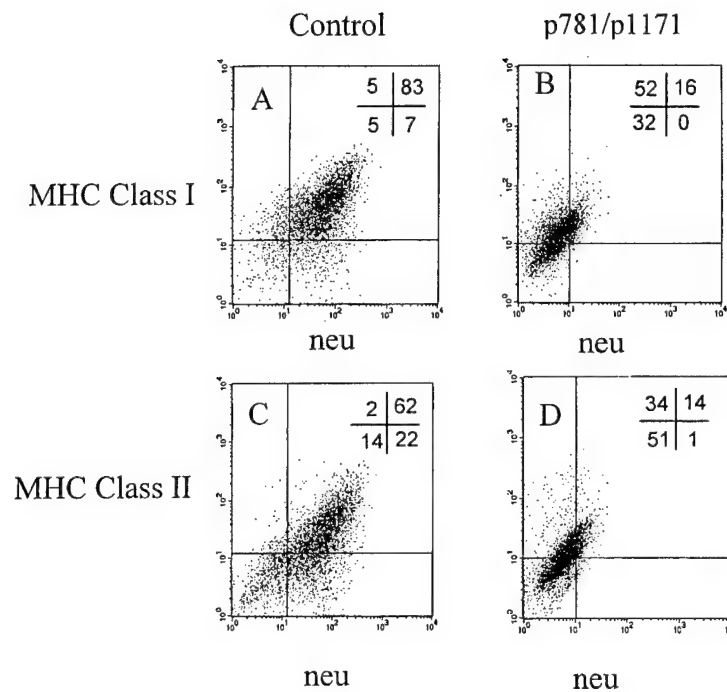


Figure 6. Antigen-negative variants can arise as a result polyclonal T cell infusions Animals were infused with a PBS (panels A and C) or a combination of p781 and p1166 specific T cell lines (panels B and D). Tumors were examined by flow cytometry following staining with neu-FITC antibody and either MHC class I-PE or MHC class II-PE antibodies. The percent of the tumor cell population falling into each quadrant is shown in the grid associated with each panel. T cell injections resulted in downregulation of the neu and MHC molecules on the cell surface of tumors.

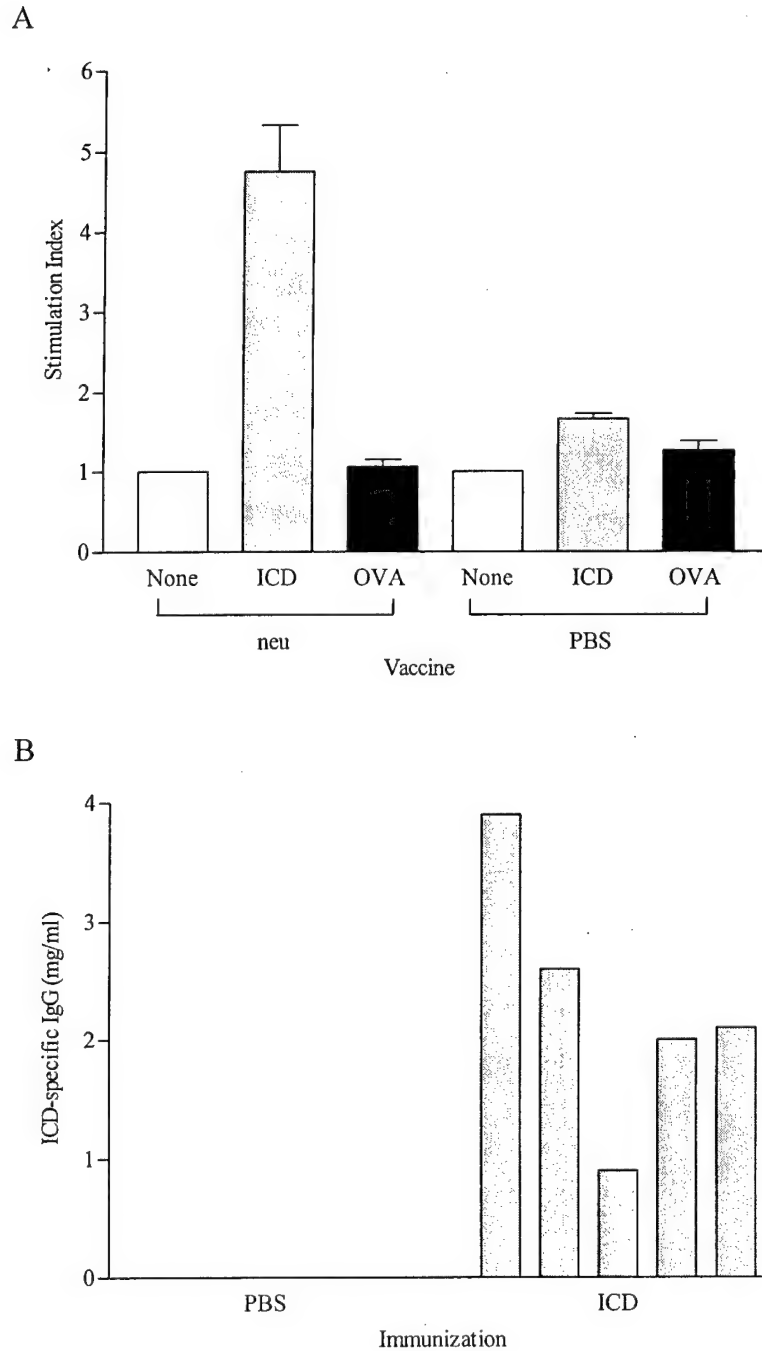
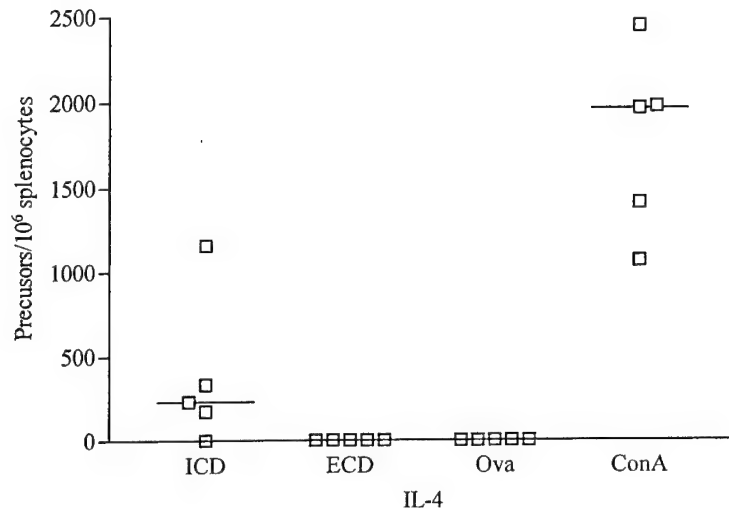


Figure 7. Neu transgenic mice can be immunized to generate significant neu specific T cell and antibody responses. A. Mice were immunized twice with recombinant ICD protein or PBS. Those animals receiving protein vaccines developed significant ICD specific T cell responses (gray bars) compared to no antigen (white bars) or ova (black bars). PBS immunized mice did not develop neu specific responses. Data is shown as the mean of 5 mice/group. B. Protein based immunization resulted in the development of significant antibody response in ICD immunized mice (n=5) compare to PBS vaccinated animals.

A



B

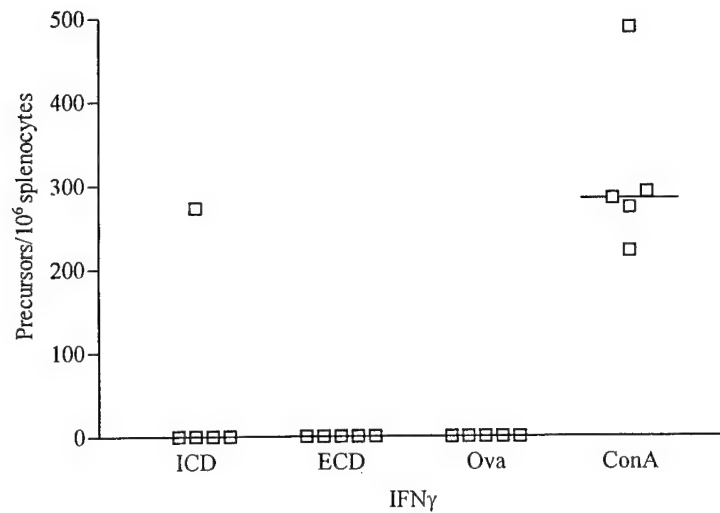


Figure 8. Th2 neu specific T cells can be elicited by neu protein immunization. A. IL-4 ELISpot demonstrates the ICD specific T cells elicited in Fig. 6A are strongly IL-4 producing. ECD protein and ovalbumin are negative controls, ConA stimulated cells are a positive control. B. ICD specific T cells elicited in Fig. 7A do not secrete IFN γ . All symbols represent the mean of 6 replicates. All means above zero are significant ($p < 0.05$).

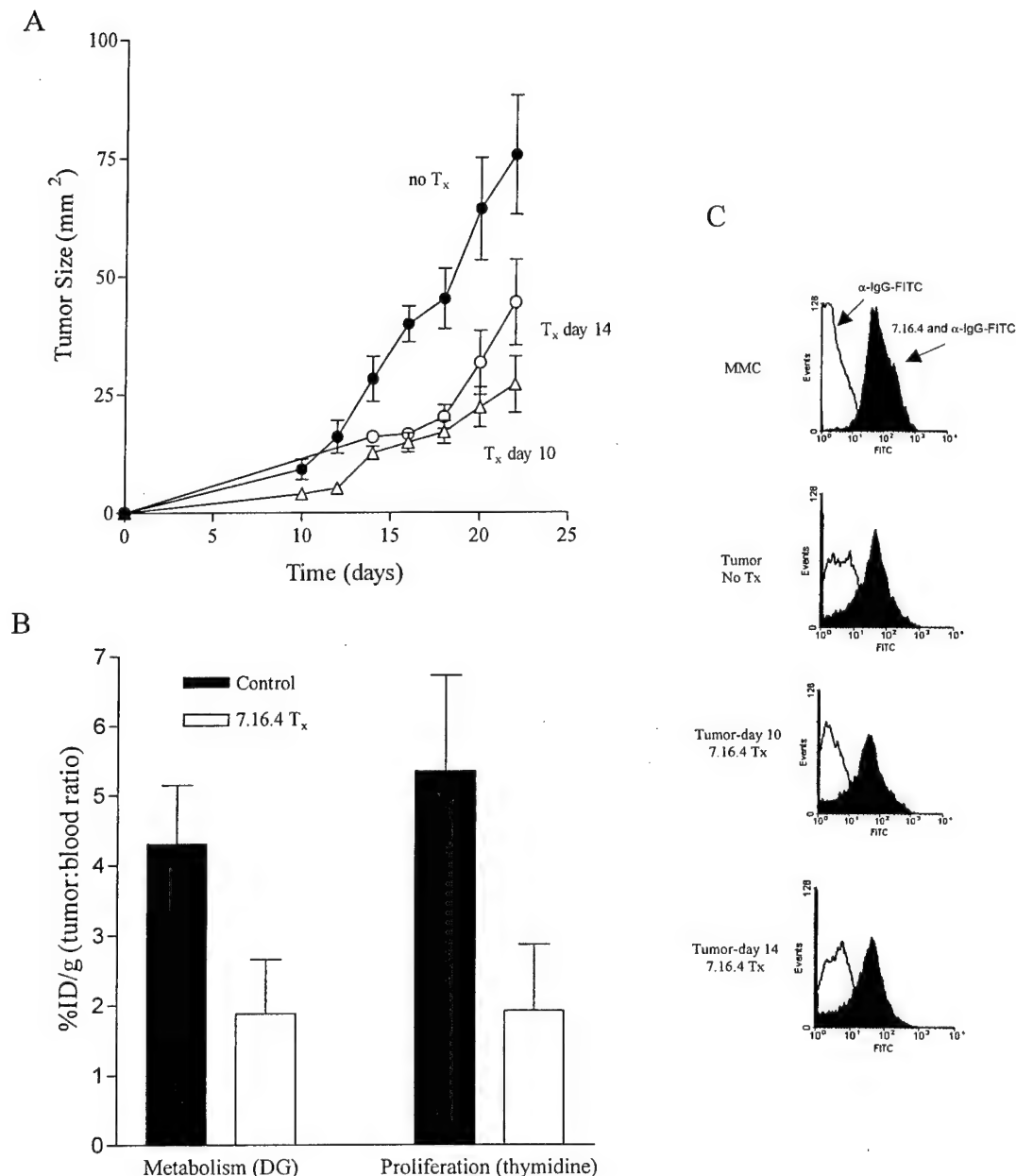


Figure 9. Anti-neu oncoprotein antibody infusions can inhibit in vivo proliferation and metabolism of tumors while without loss of cell surface neu. Animals were infused with a combination of monoclonal neu-specific antibody 7.16.4 or PBS control. Panel A: Tumor growth was measured in controls (closed circles) and animals being treated with 7.16.4 antibody (open symbols). 7.16.4 was administered to tumor bearing animals on either day 10 (T_x day 10) or day 14 (T_x day 14). Values are the mean of tumor measurements from 5 mice per group. Treated animals had significantly smaller tumors than the untreated animals after day 15 ($p < 0.05$). Panel B: Mice treated with 7.16.4 or PBS control were injected with either ³H-deoxyglucose (DG) or ¹⁴C-thymidine. Analysis of tumor uptake of radioisotopes was examined in control animals (filled bars) and treated animals (open bars) and presented as the mean % uptake of dose per gram (%ID/g) of tissue corrected for blood levels of isotope. Panel C: Tumor cell line (MMC) and tumors from control and antibody treated animals were examined for cell surface expression of neu using flow cytometry. Shown are histograms from of staining with either α-IgG-FITC alone or a combination of 7.16.4 and α-IgG-FITC. Treatment with 7.16.4 inhibits tumor cell growth without loss of neu expression at the cell surface.

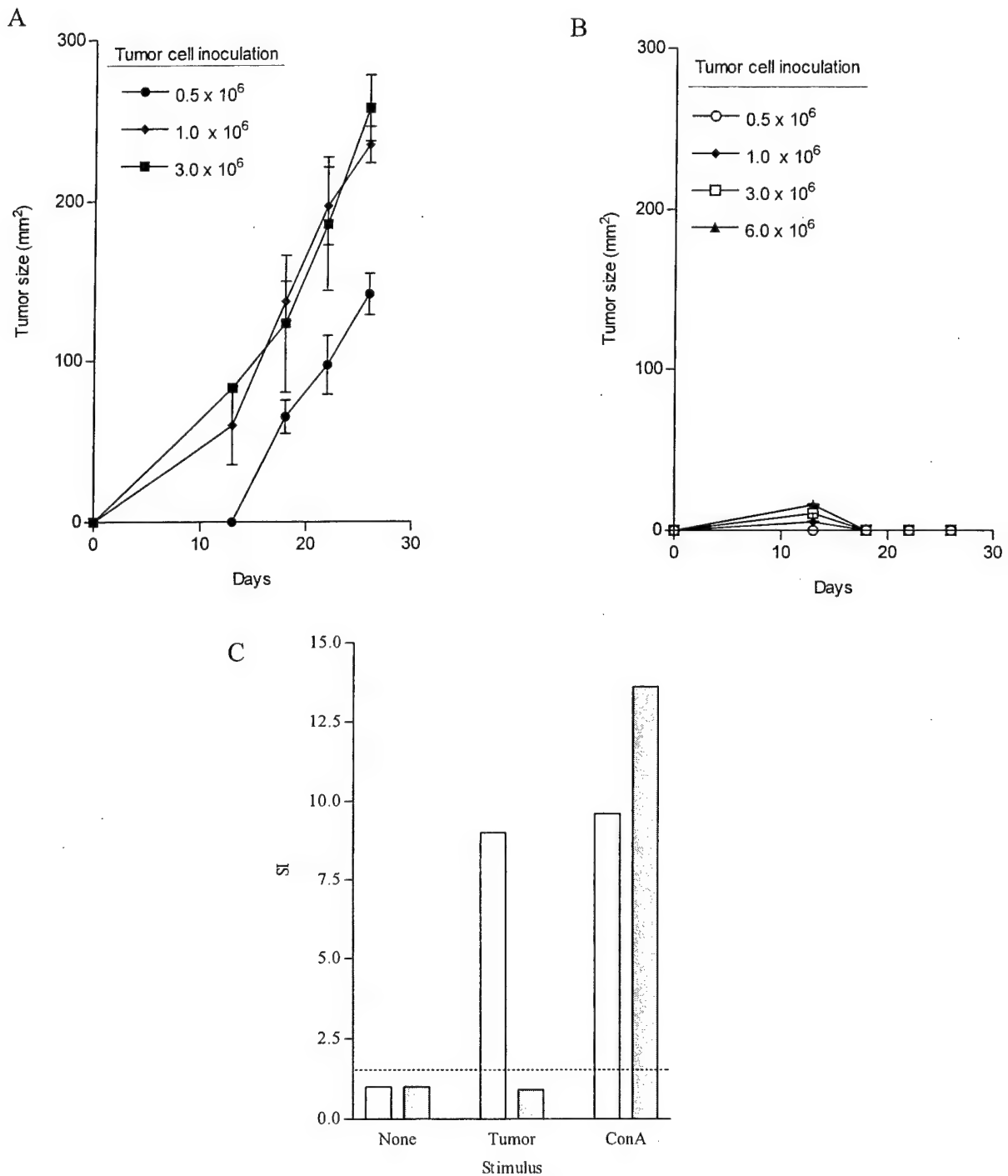


Figure 10. T cell immunity can impart a protective effect in animals where neu is a “foreign” antigen. Varying concentrations ($0.5\text{--}6.0 \times 10^6$) of MMC tumor cells were implanted into either neu-transgenic (A) or FVB parental (B) mice and monitored for growth for 28 days. Each data point is the mean (\pm sem) tumor measurements from 3 mice. (C) Splenocytes derived from neu-tg (filled bars) or FVB parental mice (open bars), injected with MMC tumor cells, were tested for proliferation against media alone (none), MMC tumor cells, or ConA blasts (ConA). Each bar is the mean of 3 determinations.

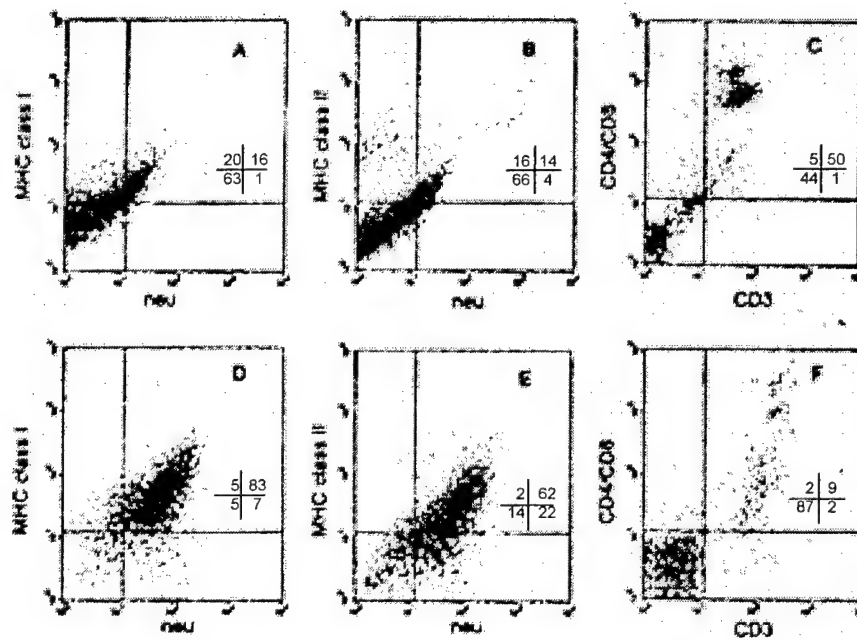


Figure 11. Down-regulation of neu, MHC class I and class II is associated with T cell infiltration into tumors in parental FVB/N mice where neu is a “foreign” protein. Neu+ MMC cells were injected in both parental FVB/N (A-C) and neu-tg mice (D-F). High doses of tumors result in tumor growth in parentals. Tumors were examined with dual staining for neu (A, B, D, E) and either MHC class I (A, D) or MHC class I (B, E). In addition, tumors were stained for the presence of T cells localized to tumor site (C, F) by triple staining for CD3, CD4, and CD8. The inset grids depicts the percentage of gated cells associated with each quadrant.

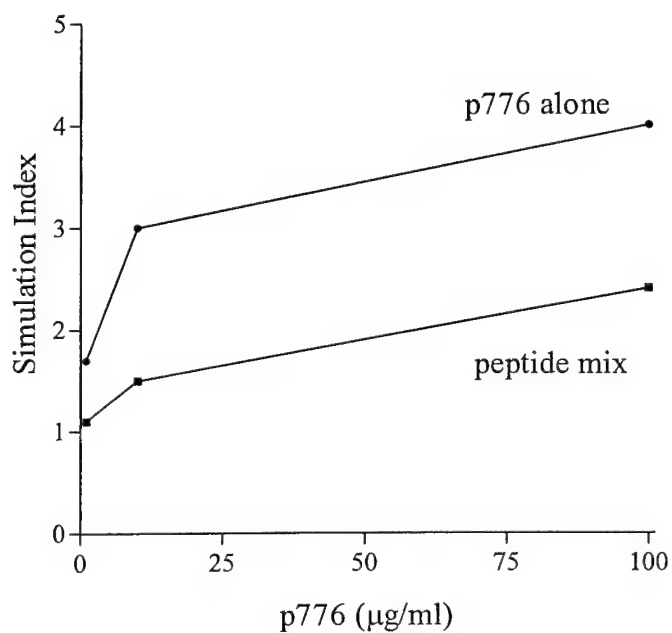


Figure 12. Expansion in the presence of a single peptide is superior to a mixture of peptides. Patient PBMC were incubated in the presence of p776 HER-2/neu peptides or a mixtures of HER-2/neu peptides that included p776. Following one in vitro stimulation, the cells were retested for proliferative activity against

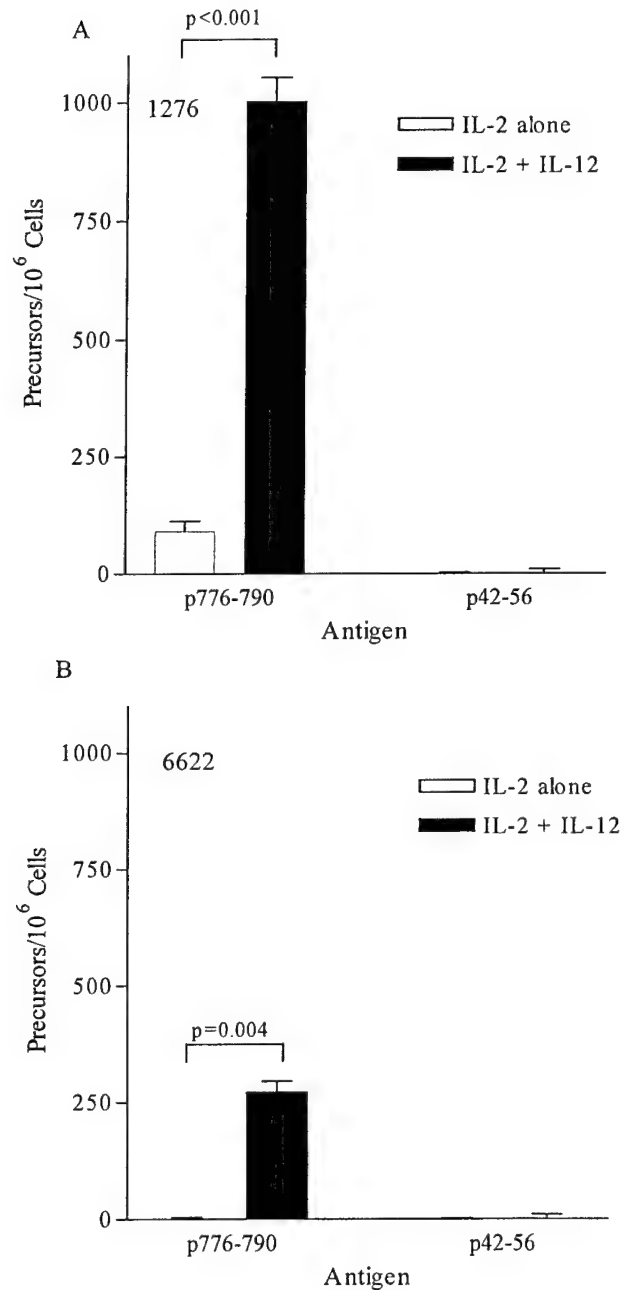


Figure 13. IL-12 used in culture to expand HER-2 specific human cells in vivo is effective in enhancing cell number. Patient 1276 (A) and 6622 (B) both were immunized with p776 and developed immunity to this peptide. After 2 IVS on peptide with IL-2 and IL-12 (gray bars) the numbers of IFN γ secreting CD4 $^{+}$ T cells were greatly enhanced compared to cultures expanded with IL-2 alone (white bars). Cultures were tested against an irrelevant peptide, p42 and demonstrated no response. Results are shown for 6 replicates/group.

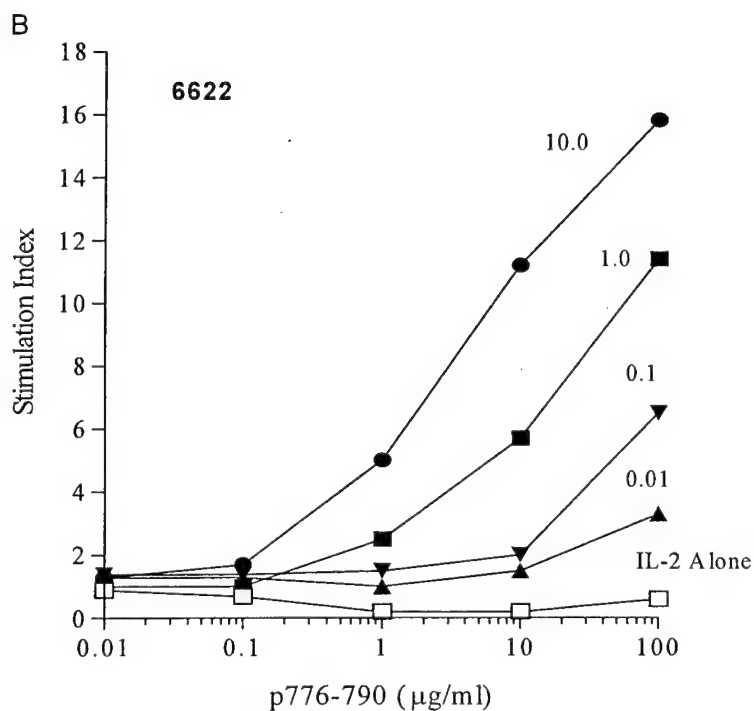
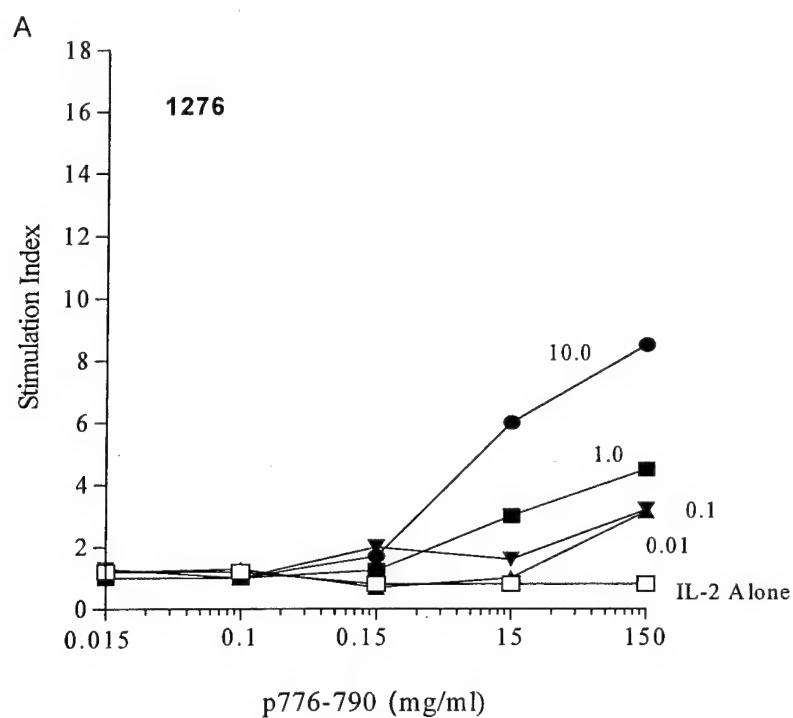


Figure 14. The dose of IL-12 used in vitro can enhance the function of neu-specific T cells. T cell cultures from patient 1276 (A) and 6622 (B) were taken through 2 IVS on peptide, IL-2, and different doses of IL-12. In both patients higher concentrations of IL-12 resulted in the generation of T cell lines which responded to lower doses of antigen.

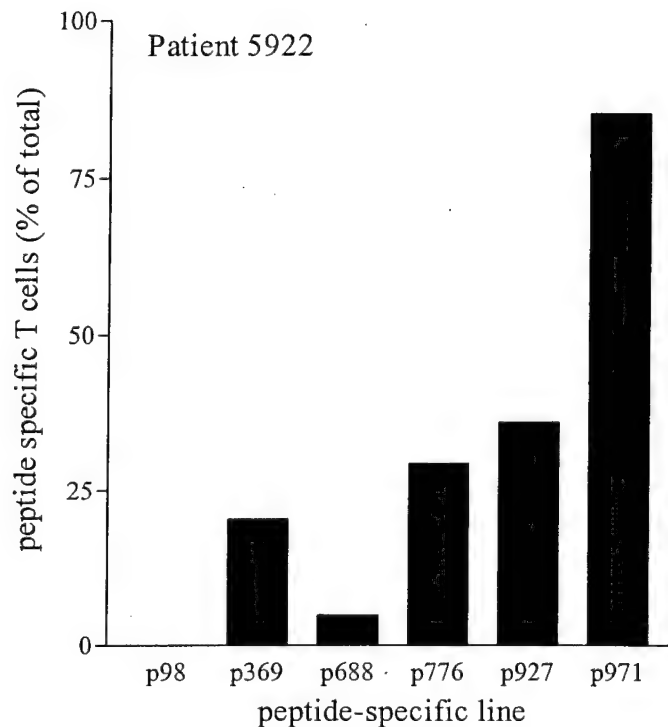


Figure 15. Human T cell lines generated from patients who have undergone active immunization can retain polyclonal characteristics and enhance precursor frequency through short term culture. Patient 5922 developed T cell responses to several peptides in her immunizing mix including epitope spreading. Data demonstrates bulk cultured lines taken through 3 IVS on peptides, IL-2, and IL-12 can retain polyclonality and enhance the specificity of responding T cells.

APPENDIX 1

Expansion of HER2/*neu*-Specific T Cells Ex Vivo Following Immunization with a HER2/*neu* Peptide-Based Vaccine

Keith L. Knutson and Mary L. Disis

Abstract

The identification and characterization of tumor antigens has facilitated the development of immune-based cancer prophylaxis and therapy. Cancer vaccines, like viral vaccines, may be effective in cancer prevention. Adoptive T-cell therapy, in contrast, may be more efficacious for the eradication of existing malignancies. Our group is examining the feasibility of antigen-specific adoptive T-cell therapy for the treatment of established cancer in the HER2/*neu* model. Transgenic mice overexpressing rat *neu* in mammary tissue develop malignancy, histologically similar to human HER2/*neu*-overexpressing breast cancer. These mice can be effectively immunized against a challenge with *neu*-positive tumor cells. Adoptive transfer of *neu*-specific T cells into tumor-bearing mice eradicates malignancy. Effective T-cell therapy relies on optimization of the ex vivo expansion of antigen-specific T cells. Two important elements of ex vivo antigen-specific T-cell growth that have been identified are (1) the preexisting levels of antigen-specific T cells and (2) the cytokine milieu used during ex vivo expansion of the T cells. Phase I clinical trials of HER2/*neu*-based peptide vaccination in human cancer patients have demonstrated that increased levels of HER2/*neu*-specific T cells can be elicited after active immunization. Initiating cultures with greater numbers of antigen-specific T cells facilitates expansion. In addition, cytokines, such as interleukin-12, when added during ex vivo culturing along with interleukin-2 can selectively expand antigen-specific T cells. Interleukin-12 also enhances antigen-specific functional measurements such as interferon-gamma and tumor necrosis factor-alpha release. Refinements in ex vivo expansion techniques may greatly improve the feasibility of tumor-antigen T-cell-based therapy for the treatment of advanced-stage HER2/*neu*-overexpressing breast malignancy.

Clinical Breast Cancer, Vol. 2, No. 1, 73-79, 2001

Key words: HER2/*neu*, Cancer vaccines, Adoptive T-cell therapy, Breast cancer, Cytokines

Introduction

The immunogenicity of tumors, such as breast cancer, reflects circumvention of tolerance to self-antigens. Increased understanding of how tolerance to self-antigens can be overcome has generated great interest in using immunotherapeutic approaches to prevent and eradicate breast malignancy.¹ One tumor-associated antigen in which there has been considerable interest is HER2/*neu*, a self-antigen overexpressed on many adenocarcinomas, including up to 30% of

breast malignancies.^{2,3} HER2/*neu* overexpression results from amplification and transcriptional upregulation of the *c-erbB-2/neu* proto-oncogene and is functionally important in tumor development and growth. In some subsets of breast cancer, HER2/*neu* overexpression correlates with a poorer prognosis.⁴

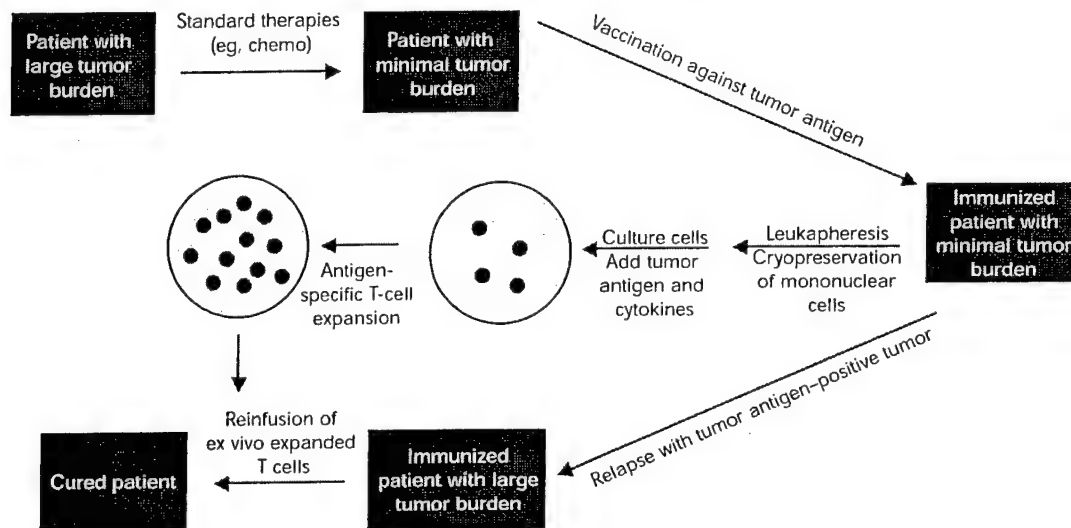
T-cell-mediated immunity directed against HER2/*neu* also exists in patients with HER2/*neu*-overexpressing cancer, suggesting that immunomodulatory intervention to enhance this immunity is possible.⁵⁻⁷ Our laboratory is actively investigating the immunologic response to HER2/*neu* following vaccination with peptide-based vaccines.^{8,9} In addition to the potential role of a vaccine in protection against subsequent tumor growth, these antigen-specific T cells could represent a source of cells for ex vivo expansion and ultimate use in adoptive T-cell-based immunotherapies targeting breast cancer (Figure 1).

Division of Medical Oncology, The University of Washington, Seattle, WA

Submitted: Aug. 16, 2000; Revised: Nov. 12, 2000; Feb. 12, 2001; Accepted: March 5, 2001

Address for correspondence: Keith L. Knutson, PhD, Division of Oncology, University of Washington, 1959 NE Pacific Street, HSB BB1321, Box 356527, Seattle, WA 98195-6527 Fax: 206-685-3128; e-mail: kknutson@u.washington.edu

Figure 1 Hypothetical Model of Antigen-Specific Adoptive T-Cell Therapy



Methods

Immunization, Tumor Challenge, and Antigen-Specific Adoptive T-Cell Therapy in the *neu*-Transgenic Mouse Model

The intracellular domain (ICD) peptide-based vaccine consisted of the rat *neu* peptides spanning the amino acid positions 781-795, 788-802, 932-948, and 1171-1185. These peptides were predicted to elicit *neu*-specific T-cell immunity.¹⁰ The *neu*-transgenic (*neu*-Tg) mice were immunized with peptides at a final concentration of 100 µg each in a total volume of 200 µL, including adjuvant. The animals underwent two immunizations 14-16 days apart. Control animals received adjuvant alone. Mice were challenged with tumor at indicated times using subcutaneous injection of 2 x 10⁶ purified syngeneic tumor cells derived from spontaneously occurring mammary tumors. T cells used for adoptive transfer were derived from spleen of control mice or mice that had been previously vaccinated with the ICD peptide-based vaccine. These T cells were either used directly or cultured for 10 days in the presence of the same peptides used for vaccination with periodic additions of interleukin-2 (IL-2).

Patient Vaccinations

Patients were enrolled into a Food and Drug Administration-approved phase I clinical trial evaluating the toxicity and immunogenicity of a HER2/*neu* peptide-based vaccine, as previously described.⁹ Briefly, the patients were immunized monthly for 6 months with a HER2/*neu* peptide vaccine consisting of putative HER2/*neu* peptides spanning the amino acids 369-384, 688-703, and 971-984. Each peptide contained within its sequences the human leukocyte antigen

(HLA)-A2 binding peptides, p369-377, p689-697, and p971-979, respectively.

Generation and Expansion of Human T-Cell Lines and Clones

Antigen-specific T-cell lines and clones were generated by culturing patient peripheral blood mononuclear cells (PBMC) in the presence of HER2/*neu* peptide, as previously described.⁹ Briefly, for the generation and expansion of HER2/*neu*-specific T-cell lines, PBMCs were cultured in 1 µM each of the HER2/*neu* HLA-A2 peptide, p369-377. For cloning, the T-cell lines were diluted to achieve approximately 0.3 viable cells/200 µL and plated onto 96-well plates in culture medium. Peptide-pulsed, irradiated autologous PBMCs (2.0 x 10⁵) were added to each well in the presence of IL-2. The clones were eventually expanded and carried using IL-2 and peptide-pulsed, irradiated autologous Epstein-Barr virus (EBV)-transformed B-lymphoblastic cells (BLCL).

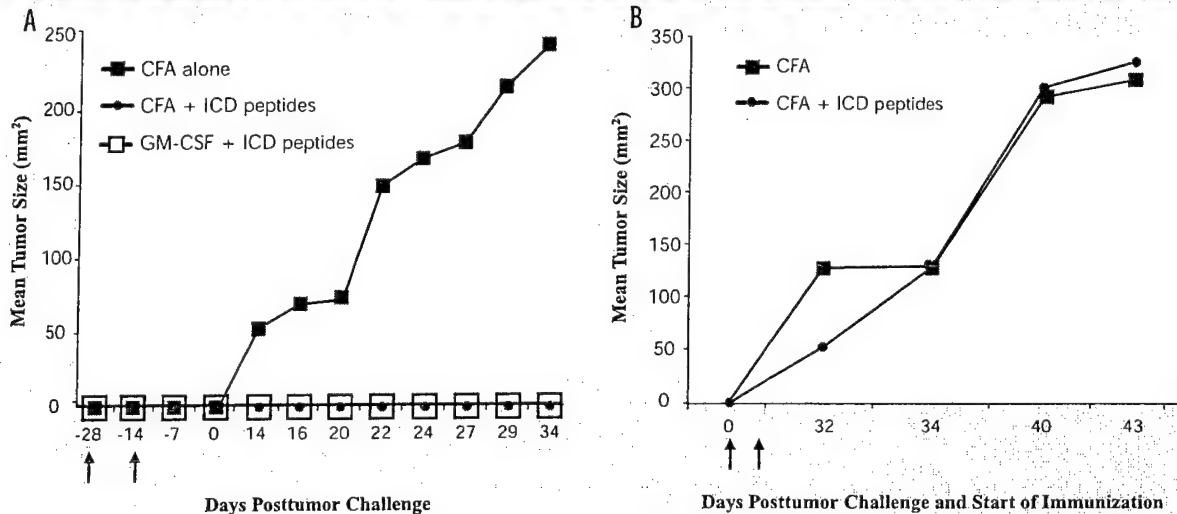
Chromium-Release Assays

Cytolytic activity was measured using standard chromium-release assays. Targets used were peptide-pulsed BLCLs, which were labeled with chromium-51 for 1-2 hours prior to use. Killing of target cells is detected as the release of chromium-51. The cytolytic reaction was carried out for 4 hours at 37°C, after which the amount of chromium-51 released into the cell culture media was assessed.

Enzyme-Linked Immunosorbent Spot

An enzyme-linked immunosorbent spot (ELISPOT) assay was used to determine frequencies of peptide-specific CD8 T

Figure 2 *Neu* Peptide Immunization Protects Against Tumor Challenge but Is Ineffective in Mediating Regression of Existing Disease



Panel A: The tumor size (mm²) was measured at the indicated times after challenge. The mice were challenged with 10⁶ cells of the 4T1 cell line. The mice were immunized with CFA alone, CFA + ICD peptides, or GM-CSF + ICD peptides. The tumor size was measured at the indicated times after challenge. Panel B: The tumor size (mm²) was measured at the indicated times after challenge. The mice were challenged with 10⁶ cells of the 4T1 cell line. The mice were immunized with CFA alone or CFA + ICD peptides. The tumor size was measured at the indicated times after challenge.

lymphocytes, as previously described.^{9,11} T cells are activated *in vitro* by being cocultured with antigen and plated onto nitrocellulose 96-well plates coated with anti-interferon-gamma antibody. The cells are incubated at 37°C for 24 hours and the interferon-gamma (IFN- γ) locally released during the incubation period is captured nearby the cell. Following the incubation period, the captured IFN- γ is detected with another secondary antibody. Binding of the secondary antibody is detected colorimetrically, and the resulting spots, representing individual cells, are enumerated. The number of antigen-specific spots is determined by subtracting the spots obtained in no antigen wells from the number of spots obtained in the presence of antigen.

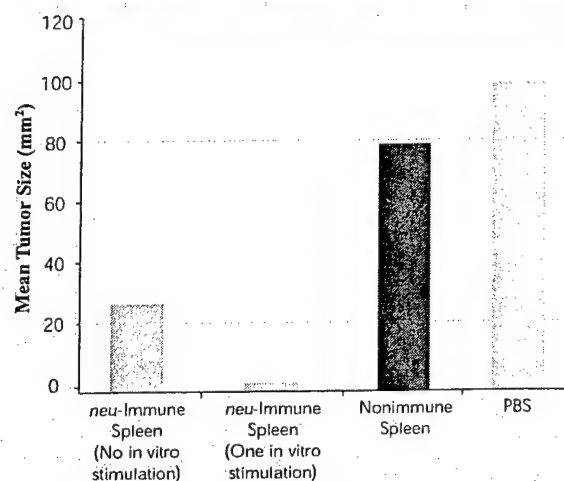
Differences in the Clinical Role of Cancer Vaccines vs. Adoptive T-Cell Therapy

It is our view that the major role of cancer vaccines likely lies in the cancer prevention rather than as a treatment modality in eradicating established malignancy. This is analogous to viral vaccines, which are administered as a prophylactic to prevent disease (eg, influenza) but not to cure it. This observation can be illustrated in an animal model of breast cancer. The *neu*-Tg mouse is an FVB mouse strain genetically engineered to overexpress rat *neu* specifically in mammary tissue.¹² Female mice spontaneously develop mammary tumors after a long latency that histologically resembles human breast carcinoma, including *neu* overexpression. In these transgenic mice, rat *neu* is a self-antigen. In previous studies, we identified several putative

major histocompatibility complex (MHC) class II binding helper peptides of rat *neu*¹⁰ and found that immunization of rats with *neu* peptides elicited *neu*-specific T-cell immunity *in vivo*. These peptides were then used for immunization in the *neu*-Tg mouse model, where it was also observed that mice developed *neu*-specific T-cell responses. In tumor protection studies, we observed that two vaccinations with *neu* peptides, derived from the ICD, were adequate for efficient rejection of tumor cells implanted subcutaneously after immunization (Figure 2A). In contrast, the mice were unable to reject tumor if vaccination was begun concurrently with tumor inoculation (Figure 2B). Thus, vaccination protects *neu*-Tg mice against future tumor challenge.

The primary purpose of adoptive T-cell therapy is to augment T-cell responses over and above that achievable by vaccination alone.¹³ Vaccination itself can increase the number of immune T cells capable of recognizing and responding to antigen. While repeated vaccination increases the number of immune effectors, eventually a plateau is reached after which further immunizations have little to no impact in further increasing the number of tumor-specific T cells.¹³ Adoptive T-cell therapy allows levels of immunity to be achieved that could potentially mediate an antitumor response, and adoptive transfer of T cells can result in the infused cells representing a large fraction (eg, 1/2) of the host's lymphocytes.¹³

Although the efficacy of administering high numbers of T cells to treat cancer in animal models is well established, in humans such attempts have met with limited success. Cancers that have been successfully treated by adoptive T-cell transfer include the EBV-related disorders, immunoblas-

Figure 3 Adoptive Transfer of *neu*-Specific T Cells Inhibits Growth of *neu*-Overexpressing Tumors In Vivo

Mice were immunized with a *neu* intracellular domain (ICD) peptide-based vaccine two times 14 days apart. Spleens were harvested from immune animals and infused (no in vitro stimulation [IVS]) into tumor-bearing mice or enriched with one IVS and then infused (one IVS). Mice were implanted with 2×10^6 *neu* syngeneic tumor cells on day 1, and on day 2, mice were infused with 2×10^7 splenocytes derived from immune spleen, immune spleen with one IVS, spleen from nonimmunized syngeneic mice, or PBS alone. Data are the mean of tumor dimensions from 3 mice/group measured 30 days after implant.

tic lymphoma, and Hodgkin's disease.¹⁴⁻¹⁶ In those studies, EBV-specific cytotoxic T lymphocytes (CTL) generated ex vivo and subsequently reinfused into patients were effective in both prophylaxis and the treatment of disease. Furthermore, the infused CTLs persisted for long periods while maintaining their antiviral properties. However, the major distinction between the treatment of EBV malignancies and nonviral cancers is the presence of nonself viral antigens. In nonviral cancers, tolerance-mediated deletion of potent and robust T cells has occurred to some extent and makes the ex vivo generation of an adequate antigen-specific T-cell population a challenge. One way to overcome this challenge is to vaccinate against antigen before retrieving T cells from the patient for expansion. Vaccination can increase the number

of antigen-specific T cells in vivo.

To understand more fully eradication of HER2/*neu*-overexpressing breast cancer by adoptive T-cell therapy, we initiated studies using the *neu*-Tg mice. The data suggest that the transfer of *neu*-specific splenocytes from *neu* peptide-immunized *neu*-Tg mice into nonimmunized naive mice can eradicate preestablished *neu*-overexpressing mammary tumors (Figure 3). The ability of the splenocytes to kill tumor was greatly enhanced by increasing the number of tumor-specific T cells with cell culture (ie, ex vivo enrichment) prior to reinfusion. In contrast, splenocytes derived from naive *neu*-Tg mice were unable to prevent tumor growth, demonstrating that prior immunization of the animals was necessary to mediate the response. Therefore, a hurdle to overcome for human clinical trials of adoptive T-cell therapy is the development of techniques for ex vivo expansion of HER2/*neu*-specific T cells.

Ex Vivo Expansion of Antigen-Specific T Cells

The requirements for the ex vivo expansion of T cells that would allow the generation of a maximal number of antigen-specific T cells while retaining optimal antigen-specific function are not well understood. Two of the most important obstacles are (1) a low preexisting antigen-specific T-cell precursor population and (2) lack of an appropriate in vitro cytokine environment.¹³

The preexisting systemic level of antigen-specific T cells is important in culturing T cells. In unprimed individuals, the ex vivo expansion of HER2/*neu* antigen-specific T cells from peripheral blood is difficult because levels of these antigen-specific T cells can be extremely low. For example, isolation and expansion of HER2/*neu*-specific T cells from patients with HER2/*neu*-overexpressing breast or ovarian carcinomas is laborious and involves lengthy expansion techniques with multiple in vitro stimulations.⁶ Therefore, in order to establish T-cell lines of sufficient antigen-specificity from unprimed individuals, one would have to start by culturing very large numbers of PBMCs.¹⁷

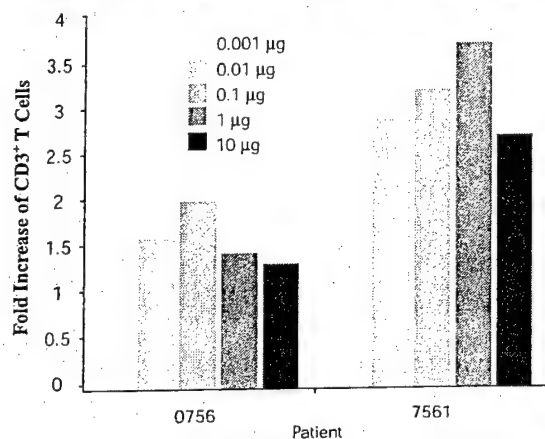
One strategy to improve expansion is to increase the frequency of antigen-specific precursors in vivo prior to ex vivo

Table 1 HER2/*neu* Peptide Immunization Results in Increased HER2/*neu* T-Cell Precursors

Donor	Preimmunization p369-377 9-mer	Postimmunization p369-377 9-mer	pflu-9-mer	TT
HLA-A2 Volunteer Donor	< 1:100,000	Not done	1:10,000	1:9000
Patient 2859	< 1:100,000	1:3800	< 1:100,000	1:22,000
Patient 0107	< 1:100,000	1:12,000	1:4500	1:4500

Patients 2859 and 0107 (both HLA-A2⁺) received the HLA-A2 vaccine containing three HER2/*neu* T-helper epitopes, p369-384, p688-703, and p971-984. P369-384 contains the HLA-A2 motif, p369-377 9-mer. Pflu-9-mer is an HLA-A2 binding motif contained within the influenza matrix protein. Precursor frequencies for an HLA-A2⁺ volunteer donor are shown for comparison. Results are presented as the ratio of p369-377 9-mer-specific T-cell precursors per number of peripheral blood mononuclear cells.

Abbreviation: HLA = human leukocyte antigen

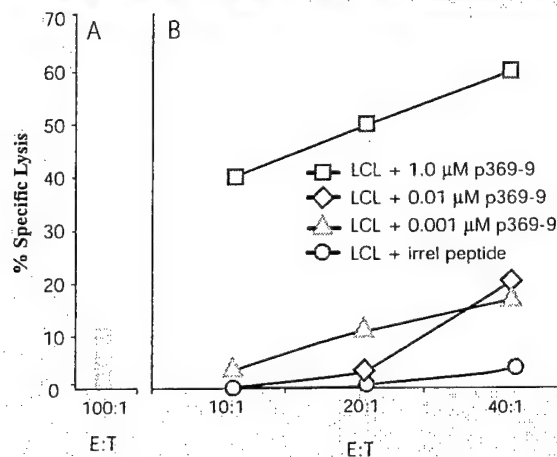
Figure 4 Concentration of HER2/*neu* Peptide p369-377 9-mer Used in In Vitro Stimulation

T lymphocytes can be expanded *ex vivo* through two in vitro stimulations with peptide antigens. Data are shown from two patients who completed a course of six immunizations with a HER2/*neu* peptide-based vaccine. Both patients were HLA-A2* and responded predominantly to p369-384 15-mer, which contains the HLA-A2 epitope, p369-377 9-mer. Cultures of peripheral blood mononuclear cells were taken through two in vitro stimulations with various concentrations of p369-377 9-mer. The graphs represent expansion of total CD3⁺ T cells over starting numbers.

expansion by vaccination. Studies from our group have shown that boosting HER2/*neu* precursor frequencies by peptide immunization has allowed us to more readily expand and clone HER2/*neu*-specific T cells from the peripheral blood of patients with HER2/*neu*-overexpressing breast cancers as compared to naive donors (K. Knutson, unpublished observations).

Phase I clinical trials were initiated in 1997 to assess both toxicity and immunogenicity of HER2/*neu* peptide-based vaccines in patients with stage III or IV breast or ovarian cancer. All of the vaccine formulations target HER2/*neu* peptides in either the ICD, extracellular domain (ECD), or both.² Granulocyte-macrophage colony-stimulating factor was used as an adjuvant,¹⁸ and the immunizations were administered intradermally monthly for 6 months. The initial trial tested three helper T-cell vaccines, each composed of three MHC class II binding peptides, 15-18 amino acids in length.^{2,9} One of the vaccine preparations (HLA-A2 vaccine) consisted of three putative T-helper epitopes of HER2/*neu*, each of which contains a complete HLA-A2 binding motif within their sequence. Vaccines using this strategy have been demonstrated to elicit both helper and cytolytic T-cell immunity.⁹

Recently, the analysis of the 19 patients who received the HLA-A2 vaccine was reported.⁹ In that study, it was observed that the majority of patients developed both HER2/*neu* peptide- and protein-specific immunity. Despite generating significant levels of immunity, the HER2/*neu* vaccine was well tolerated. Detection of HER2/*neu*-specific T cells was assessed using an IFN- γ -ELISpot assay, which has been

Figure 5 Lytic Activity of Antigen-Specific T Cells Is Enriched Following Two In Vitro Stimulations

Data shown are from patient 7561, who completed a course of six immunizations with a HER2/*neu* peptide-based vaccine. The dominant response was directed against p369-384 15-mer, which contains the HLA-A2 epitope, p369-377 9-mer (p369-9). The patient was HLA-A2*. Panel A demonstrates the level of lytic activity of T cells after one in vitro stimulation (IVS) with p369-377 9-mer. The lytic activity was examined at an effector-to-target (E:T) ratio of 100:1 against autologous, chromium-51B-labeled Epstein-Barr virus-transformed lymphoblastoid cell line (LCL) loaded with p369-377 9-mer. Activity against nonloaded LCL was < 1%. Panel B demonstrates enrichment of lytic activity of T cells after two IVS with p369-377 9-mer. Target LCL were loaded with varying concentrations of peptides. Cultures from both panels were established by IVS with 1 µM p369-384 15-mer (immunizing peptide).

Abbreviation: irrel peptide = irrelevant HLA-A2 binding peptide

shown to be a reliable method to detect antigen-specific T cells existing in the peripheral blood at levels as low as 1 in 100,000 mononuclear cells. We demonstrated that T cells specific for HER2/*neu* were increased following vaccination⁹ (Table 1), allowing us to more readily expand functional T cells in the presence of antigen (Figure 4).

The antigen-specific expansion of the T cells also enriches the cytolytic activity against HER2/*neu* (Figure 5). Furthermore, increased T-cell precursors facilitate isolation of T-cell clones. For example, we isolated 21 p369-377 peptide-specific clones from an HLA-A2 patient immunized with the HLA-A2 vaccine.¹⁹ Most of the clones (19 of 21) expressed the broad T-cell marker, CD3. While 11 of the clones expressed CD8⁺ (cytolytic T cells), nine expressed CD4⁺ (helper T cells) despite being cloned with the HLA-A2-binding 9-mer. Since 9-mer peptides in association with HLA-A predominantly regulate CD8⁺ T cells, the significance of CD4⁺ T cells responding to HLA-A2 9-mer is not well understood. Also, 19 of the clones expressed the alpha-beta T-cell receptor, and interestingly, two expressed the gamma-delta T-cell receptor.²⁰ Additionally, several of the clones secreted IFN- γ directly in response to p369-377 9-mer and could lyse HLA-A2*, HER2/*neu*-overexpressing tumor cells. These findings suggest that vaccination may have the potential to expand different HER2/*neu*-specific T-cell subsets and may eventually allow identification of T cells most effective at eradicat-

Table 2 Cytokines Used for Ex Vivo Expansion of Antigen-Specific T Cells

Cytokine	Natural Source	Function
IL-2	Activated T cells	Proliferation of T cells ¹³
		Proliferation of T cells ²²
IL-12	Phagocytes	Enhances cytolytic activity ²³
		Promotes cell-mediated immunity ²⁴
		Enhances cytokine production ²⁵
		Upregulates IL-2 receptor expression ²⁶
IL-4	CD4 ⁺ T cells	Enhances IL-12 production ²⁷
		Enhances maturation of memory T cells ²⁸
		T-cell differentiation ²⁹
IL-7	Stromal cells	T-cell survival ³⁰
		T-cell proliferation ³¹
		Proliferation of T cells ³²
IL-15	Activated T cells	Maintenance of memory T cells ³³
		Inhibition of apoptosis ³⁴

Abbreviation: IL = interleukin

ing tumor.

The cytokine environment needed to expand a functional antitumor population and sustain it in vivo has been elucidated to some degree.¹³ For example, IL-2, when added during in vitro stimulation of T cells, promotes proliferation and increased survivability of cells in vivo.¹³ However, IL-2 activates T-cell lines nonspecifically and often antigen specificity is not preserved. We have found that IL-12, when added along with IL-2, enhances both expansion and function of HER2/neu-specific T-cell lines.²¹ For example, IL-12 plus IL-2 enhances HER2/neu-specific tumor necrosis factor- α release up to sixfold greater than that observed with IL-2 only following in vitro stimulation with HER2/neu peptides. Over the past decade, other T-cell-active cytokines, such as IL-4, IL-7, and IL-15, have been identified, which may exhibit similar in vitro activities (Table 2).^{13,22-34} Studies are ongoing in our laboratory to characterize these cytokines for their utility in ex vivo expansion of HER2/neu-specific T cells.

Conclusions

Tumor antigen-targeted T-cell-based therapy is a feasible treatment strategy for HER2/neu-overexpressing malignancies. Vaccination alone most likely will not be effective as a treatment strategy for established disease but rather in pre-

venting the recurrence of micrometastatic disease. Treatment of established disease will require T-cell therapy with ex vivo-expanded tumor-specific T cells. The use of vaccination followed by carefully manipulated ex vivo antigen-mediated expansion of T cells offers the potential of increasing tumor-specific T cells to numbers greater than that which could be achieved by vaccination alone. Ex vivo expansion has been problematic and many hurdles will need to be overcome. Two important issues are (1) increasing antigen-primed T cells in vivo prior to ex vivo expansion by vaccination and (2) improved culture conditions with the use of recently identified cytokines in conjunction with IL-2. The refinement of techniques for antigen-specific T-cell therapy has ushered in a new era for the treatment of breast cancer.

Acknowledgements

This work was supported by Keith L. Knutson by a fellowship from the Department of Defense Breast Cancer Program and for Mary L. Disis by National Cancer Institute grants R01 CA75163 and R01 CA85374, The Cancer Research Treatment Foundation, and the Department of Defense Breast Cancer Program.

References

- Nanda NK, Sercarz EE. Induction of anti-self-immunity to cure cancer. *Cell* 1995; 82:13-17.
- Disis ML, Cheever MA. HER-2/neu oncogenic protein: issues in vaccine development. *Crit Rev Immunol* 1998; 18:37-45.
- Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235:177-182.
- Gusterson BA, Gelber RD, Goldhirsch A, et al. Prognostic importance of c-erbB-2 expression in breast cancer. International (Ludwig) Breast Cancer Study Group. *J Clin Oncol* 1992; 10:1049-1056.
- Disis ML, Calenoff E, McLaughlin G, et al. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res* 1994; 54:16-20.
- Fisk B, Blevins TL, Wharton JT, et al. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* 1995; 181:2109-2117.
- Peoples GE, Goedegebuure PS, Smith R, et al. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci U S A* 1995; 92:432-436.
- Disis ML, Grabstein KH, Sleath PR, et al. Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin Cancer Res* 1999; 5:1289-1297.
- Knutson KL, Schiffman K, Disis ML. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *J Clin Invest* 2001; 107:477-484.
- Disis ML, Gralow JR, Bernhard H, et al. Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self-protein. *J Immunol* 1996; 156:3151-3158.
- McCutcheon M, Wehner N, Wensky A, et al. A sensitive ELISPOT assay to detect low-frequency human T lymphocytes. *J Immunol Methods* 1997; 210:149-166.
- Guy CT, Webster MA, Schaller M, et al. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A* 1992; 89:10578-10582.
- Cheever MA, Chen W. Therapy with cultured T cells: principles revisited. *Immunol Rev* 1997; 157:177-194.
- Roskrow MA, Suzuki N, Gan Y, et al. Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. *Blood* 1998; 91:2925-2934.
- Rooney CM, Smith CA, Ng CY, et al. Use of gene-modified virus-spe-

- cific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* 1995; 345:9-13.
16. Heslop HE, Ng CY, Li C, et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med* 1996; 2:551-555.
17. Carbone FR, Moore MW, Sheil JM, et al. Induction of cytotoxic T lymphocytes by primary in vitro stimulation with peptides. *J Exp Med* 1988; 167:1767-1779.
18. Disis ML, Bernhard H, Shiota FM, et al. Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood* 1996; 88:202-210.
19. Knutson KL, Crosby P, Disis ML. Isolation of HER-2/neu (HER2) specific T cell clones from a cancer patient following immunization with a HER2 peptide vaccine. *Prom Am Assoc Cancer Res* 1999; 40:310 (Abstract).
20. Knutson KL, Disis ML. Isolation of HER-2/neu (HER2) specific gamma-delta TCR T cell clones from an HLA-A2 ovarian cancer patient immunized with a peptide-based vaccine. *FASEB* 2000; 14:A1006 (Abstract).
21. Knutson KL, Disis ML. IL-12 enhances in vitro expansion and function of peptide-specific T lymphocytes. *Proc Am Assoc Cancer Res* 2000; 41:193 (Abstract).
22. Gately MK, Desai BB, Wolitzky AG, et al. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J Immunol* 1991; 147:874-882.
23. McFarland EJ, Harding PA, MaWhinney S, et al. In vitro effects of IL-12 on HIV-1-specific CTL lines from HIV-1-infected children. *J Immunol* 1998; 161:513-519.
24. Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 1994; 84:4008-4027.
25. Gerosa F, Paganin C, Peritt D, et al. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. *J Exp Med* 1996; 183:2559-2569.
26. Casey LS, Lichtman AH, Boothby M. IL-4 induces IL-2 receptor p75 beta-chain gene expression and IL-2-dependent proliferation in mouse T lymphocytes. *J Immunol* 1992; 148:3418-3426.
27. Abdi K, Herrmann SH. CTL generation in the presence of IL-4 is inhibited by free p40: evidence for early and late IL-12 function. *J Immunol* 1997; 159:3148-3155.
28. Roth AD, Dupuis S, Alberto P. Human recombinant IL-4 decreases the emergence of non-specific cytolytic cells and favours the appearance of memory cells (CD4+CD45RO+) in the IL-2-driven development of cytotoxic T lymphocytes against autologous ovarian tumour cells. *Clin Exp Immunol* 1995; 101:362-368.
29. Hofmeister R, Khaled AR, Benbernou N, et al. Interleukin-7: physiological roles and mechanisms of action. *Cytokine Growth Factor Rev* 1999; 10:41-60.
30. Marrack P, Mitchell T, Bender J, et al. T-cell survival. *Immunol Rev* 1998; 165:279-285.
31. Takashima A, Matsue H, Bergstresser PR, et al. Interleukin-7-dependent interaction of dendritic epidermal T cells with keratinocytes. *J Invest Dermatol* 1995; 105:50S-53S.
32. Hasan MS, Kallas EG, Thomas EK, et al. Effects of interleukin-15 on in vitro human T cell proliferation and activation. *J Interferon Cytokine Res* 2000; 20:119-123.
33. Zhang X, Sun S, Hwang I, et al. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* 1998; 8:591-599.
34. Bulfone-Paus S, Ungureanu D, Pohl T, et al. Interleukin-15 protects from lethal apoptosis in vivo. *Nat Med* 1997; 3:1124-1128.

APPENDIX 2

Clonal Diversity of the T-Cell Population Responding to a Dominant HLA-A2 Epitope of HER-2/neu After Active Immunization in an Ovarian Cancer Patient

Keith L. Knutson, and Mary L. Disis

ABSTRACT: Natural antigen processing and presentation of antigen is thought to be important for the generation of a broad functional repertoire of antigen-specific T cells. In this study, the T-cell repertoire to an immunodominant human leukocyte antigen A2 (HLA-A2) binding peptide epitope of HER-2/neu, p369-377, was examined in a patient following immunization with a peptide-based vaccine consisting of helper peptides encompassing HLA-A2 peptide epitopes. The responding T-cell repertoire generated was both phenotypically and functionally diverse. A total of 21 p369-377 clones were generated from this patient. With the exception of two clones, all clones were CD3⁺. Sixteen of the clones were CD8⁺/CD4⁻. Five of the clones were CD4⁺/CD8⁻, despite being generated with an HLA-A2 binding peptide. Nineteen of 21 of clones expressed the $\alpha\beta$ -TCR. The remaining two clones expressed the $\gamma\delta$ T-cell response (TCR). Selected $\alpha\beta$ -TCR clones, both CD8⁺ and CD4⁺, could

lyse HLA-A2 transfected HER2 overexpressing tumor cells and p369-377-loaded B-lymphoblastic cell line. In addition to their lytic capabilities these clones could be induced to produce interferon- γ (IFN- γ) specifically in response to p369-377 peptide stimulation. The 2 $\gamma\delta$ -TCR clones expressed CD8 and lysed HLA-A2⁺ HER-2/neu⁺ tumor cells, but not HLA-A2⁻ HER-2/neu⁺ tumor cells. One of $\gamma\delta$ -TCR clones also released IFN- γ directly in response to p369-377 stimulation. These results suggest that a tumor antigen TCR, directed against a specific epitope, can be markedly polyclonal at multiple levels including CD4/CD8 and TCR. *Human Immunology* 63, 000 (2002). © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: vaccination; T lymphocytes; CTL; tumor immunity; $\gamma\delta$ T cells

ABBREVIATIONS

BLCL B-lymphoblastic cell line
TCR T-cell receptor

CTL cytolytic T lymphocyte

INTRODUCTION

One of the most common vaccination strategies used to generate T cells specific for tumor antigen is to use peptides formulated to bind directly to human leukocyte antigen (HLA) molecules eliminating the need for intracellular processing and presentation of antigen. Vaccination with peptides is advantageous compared with other methods, such as using DNA or proteins, for several reasons including the fact that peptides are easily con-

structed and can be chosen to target specific T-cell subsets, such as cytolytic T cells (CTL) [1-3]. Vaccination using HLA class I binding peptides have been tested most often because of the availability of HLA class I sequence information and the observations that CTL are critical for antitumor immunity.

Some clinical studies using vaccination with HLA class I binding peptides have reported that peptide-specific precursor frequencies can become elevated, but that these peptide-specific T cells may not recognize naturally processed antigen [4]. The lack of recognition of antigen presented in the major histocompatibility complex (MHC) may be due to the fact that the peptides, usually constructed from motif-based algorithms, were not naturally processed. Alternatively, the high pharmacologic levels of peptides usually administered in a vac-

From the Division of Oncology, Department of Medicine, University of Washington, Seattle, WA, USA.

Address reprint requests to: Dr. K.L. Knutson, Division of Oncology, University of Washington, 1959 NE Pacific Street, HSB BB1321, Box 356527, Seattle, WA 98195-6527; Tel: (206) 221-5417; Fax: (206) 685-3128; E-mail: kknutson@u.washington.edu.

Received October 22, 2001; revised March 1, 2002; accepted March 14, 2002.

Human Immunology 63, 000 (2002)

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Published by Elsevier Science Inc.

0198-8859/02/\$-see front matter
PII S0198-8859(02)00401-9

cine could have elicited a T-cell repertoire that cannot respond to the low levels of peptides presented naturally. Thus, it is becoming apparent that natural levels of presentation of peptide antigen, delivered through antigen processing pathways, may be better suited to generate an immune response when using peptide-based vaccines. The levels of endogenously processed antigen presented by antigen presenting cells is a function of many factors including the antigen load as well as the types and amounts of proteasomes present [5]. Several strategies have been developed to deliver peptides to the inside of the cell where natural presentation can be achieved, including mini-gene epitope delivery [6] or fusing the epitopes with molecules designed to deliver the peptides to the endosomal pathway, such as lysosomal membrane associated protein [7]. Another strategy is to deliver the HLA class I peptides that are fully contained within longer helper epitopes, which can result in natural presentation of the encompassed class I peptides [8].

In this study, we examined the phenotypic and functional diversity of the T-cell repertoire to HER-2/neu peptide p369-377 in a HLA-A2 patient with a HER-2/neu-overexpressing ovarian cancer. The patient had been previously immunized with a HER-2/neu helper peptide vaccine, which contained the helper peptide p369-386 [8]. This helper peptide encompassed the HLA-A2 peptide, p369-377. Therefore, the resulting p369-377-specific T-cell repertoire produced *in vivo* was a function of natural processing of antigen.

MATERIALS AND METHODS

Patient

The patient was enrolled in a Phase I HER-2/neu peptide-based vaccine trial approved by the University of Washington's Human Subjects Division and the United States Food and Drug Administration, and had received definitive conventional therapy for her disease [8]. This Phase I clinical trial was designed to evaluate safety and immunologic responses to the vaccine. Furthermore, all of the patients had either no evidence of disease or minimal residual stable disease. Clinical responses to vaccine were not possible unless patients were followed for an extended time to relapse. The patient signed a protocol-specific consent and received monthly vaccinations with three 15 amino acid (15-aa) HER-2/neu-derived peptides (p369-386, p688-703 and p971-984) containing within each the putative HLA-A2 binding motifs p369-377 [9], p689-697 [10], and p971-979 [11]. The vaccine preparation was prepared and delivered as previously described [8]. The patient underwent peripheral blood draws prior to and 30 days following each vaccination for immunologic monitoring. Leukapheresis

was obtained 30 days following the final vaccination for the generation of T-cell clones.

Materials

The following peptides used in this study, either for immunization or *in vitro* use, were as follows: HER-2/neu peptides, p369-386, KIFGSLAFLPESFDGDPA [12], p688-703, RRLQETELVEPLTPS [12], p971-984, ELVSEFSRMARDPQ [12], p369-377, KIFGSLAFL, p1066-1074, SEEEAPRSP. All peptides used for *in vitro* immunologic assays were manufactured either by United Biochemical Inc. (Seattle, WA, USA) or Multiple Peptide Systems (San Diego, CA, USA) and all were > 95% pure as assessed by HPLC and mass-spectrometric analysis. Ficoll/Hypaque was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI-1640, HBSS, and PBS were purchased from Life Technologies (Rockville, MD, USA), and EHAA-120 was purchased from Biofluids (Rockville, MD, USA). The [³H] thymidine and [⁵¹Cr] sodium chromate were purchased from NEN Life Science Products (Boston, MA, USA), human AB+ serum from Valley Biomedical, Inc. (Winchester, VA, USA), sterile nitrocellulose-backed microfiltration 96-well plates from Millipore Corp (Bedford, MA, USA), and streptavidin-alkaline phosphatase and AP-colorimetric reagents were from BioRad (Hercules, CA, USA). Purified anti-IFN- γ (clone # 1-D1K) and biotin-conjugated anti-IFN- γ (clone # 7-B6-1) were purchased from Mabtech AB (Nacka, Sweden). Anti-CD8-FITC, anti-CD4-PE, anti- $\gamma\delta$ -TCR, and anti- $\alpha\beta$ -TCR antibodies were purchased from Pharmingen (San Diego, CA, USA). HLA testing was performed by the Puget Sound Blood Bank (Seattle, WA, USA).

Cell Lines

Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines (BLCL) were produced from peripheral blood mononuclear cells (PBMC) using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, VA, USA). The HER-2/neu-overexpressing cell lines SKOV3 and SKOV3-A2, and BLCLs were maintained in RPMI-1640 with L-glutamine, penicillin, streptomycin, 2-mercaptoethanol, and 10% fetal calf serum. The SKOV3-A2 tumor cells are the same as SKOV3 tumor cells, except that they are stably transfected with a vector encoding HLA-A2 [13].

Preparation of PBMC and Cloning of Peptide-Specific T Cells

PBMC were isolated by density gradient centrifugation as previously described [12]. Cells were analyzed immediately or aliquoted and cryopreserved in liquid nitrogen in freezing media (90% fetal bovine serum and 10%

dimethylsulfoxide) at a cell density of 25 to 50×10^6 cells/ml until use. Antigen-specific T-cell clones were generated by culturing 25×10^6 PBMC in T25 tissue culture flasks in 20 ml of T-cell medium. The HLA-A2 peptide, p369-377, was added to the flasks to $1 \mu\text{M}$. The flasks were incubated at 37°C and $5\% \text{CO}_2$. On day 3 and every other subsequent day, IL-2 was added to 5 U/ml. On day 10, *in vitro* stimulation was performed with peptide-pulsed, irradiated autologous PBMC. The cultures were further incubated for an additional 10 days with periodic IL-2 administration. T-cell clones were derived from the T-cell lines after two *in vitro* stimulations. For cloning, bulk cultures were diluted to achieve approximately 0.3 viable cells/200 μl and plated onto four flat-bottom 96-well plates in complete medium. Peptide-pulsed, irradiated autologous PBMC (2.0×10^5) were added to each well in the presence of 50 U/ml IL-2. The wells were then tested for lytic activity in a ^{51}Cr release assay using 50 μl of cells from each well after 14 days. Positive wells were identified as those having specific activity of 5% or greater. The positive wells were transferred to new 96-well plates and subsequently restimulated with peptide-pulsed, irradiated autologous BLCL.

The cultures were eventually expanded and carried using IL-2 and peptide-pulsed, irradiated autologous BLCL.

T-Cell Proliferation Assays

HER-2/neu-specific T-cell proliferative responses were measured at baseline and at the end of the study. T-cell proliferation was assessed using a modified limiting dilution assay designed for detecting low frequency lymphocyte precursors based on Poisson distribution as previously described [12, 14]. HER-2/neu peptide-specific T-cell responses were measured at baseline and at the time of the final vaccination using freshly prepared PBMC. PBMC were prepared by Ficoll-Paque (Pharmacia AB, Uppsala, Sweden) centrifugation and resuspended in media consisting of equal parts of EHAA 120 (Biofluids, Inc.) and RPMI 1640 (Gibco, Grand Island, NY, USA) with L-glutamine, penicillin/streptomycin, β -2 ME, and 10% AB serum (ICN Flow, Costa Mesa, CA). PBMC were cocultured with 50 $\mu\text{g}/\text{ml}$ of the various individual HER-2/neu peptides. Specifically, 2×10^5 PBMC/well were plated into 96-well round bottom microtiter plates (Costar, Cambridge, MA, USA) with antigen at 37°C in an atmosphere of $5\% \text{CO}_2$ for 5 days. All antigens were tested in 24-well replicates. Eight hours before termination of culture, each well was pulsed with 1 μCi ^3H -thymidine (New England Nuclear, Wilmington, DE). The cultures were then harvested onto glass fiber filters and the incorporated radioactivity was measured with a Microbeta 1450

scintillation counter (Wallac). Peripheral blood T-cell response data presented here is expressed as a standard stimulation index (SI), which is defined as the mean of all 24 experimental wells divided by the mean of the 24 control wells (no antigen). An age-matched control population of 30 volunteer blood donors was analyzed similarly (data not shown). No volunteer donor had a response to HER-2/neu peptides. The mean and 3 standard deviations of the volunteer donor responses to all antigens (SI of 1.98) established a baseline, therefore an SI of > 2 was considered consistent with an immunized response.

Enzyme-Linked Immunosorbant Spot

An enzyme-linked immunosorbent spot (ELISpot) assay was used to determine precursor frequencies of peptide-specific T lymphocytes as previously described with some minor modifications [8]. On day 1, 2.5×10^4 viable cells, as determined by trypan blue staining were plated, in quadruplicate, into 96-well, anti-IFN- γ -coated nitrocellulose plates in 100 μl media. Because at 10 days following restimulation some irradiated PBMC and BLCL can still exclude trypan blue, the viable cell counts do not reflect the true numbers of each antigen-specific clonal population. Thus, our ELISpot analysis is only able to measure the numbers of cells responding to antigen and not the proportion of the antigen-specific T-cell clones that are responding with IFN- γ release. The cells were stimulated with 100 μl of media containing 2.0×10^5 autologous, irradiated (3000 rads) BLCLs prepulsed (1 h, RT) with or without antigen (10 $\mu\text{g}/\text{ml}$). The cells were further incubated for 20 h at 37°C and detection of bound IFN- γ was performed as previously described [8].

[^{51}Cr]-Release Assays

Cytolytic activity was measured using standard 4-h [^{51}Cr]-release assays as previously described [8]. The percent specific activity was calculated using the following equation: % specific lysis = (sample well release - basal release)/(detergent release - basal release).

Flow Cytometry

Clones were harvested and washed in FACS staining buffer (PBS containing 20-mM glucose and 0.5% BSA). Cells were stained in 50- μl FACS staining buffer containing control antibody, anti-CD4, anti-CD8, anti- $\alpha\beta$ -TCR, anti- $\gamma\delta$ -TCR, or anti-CD3, for 1-2 h at 4°C . Following two washes the cells were fixed in PBS containing 1% paraformaldehyde and analyzed by flow cytometry. Data presentation was completed using CellQuest flow cytometry software (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA).

RESULTS

Immunity to HER-2/neu peptide p369-386 is generated as a result of vaccination with helper peptides in an HLA-A2⁺ ovarian cancer patient. T-cell proliferative responses were measured against 15-aa HER-2/neu peptides prior to, during, and following the vaccination series. As illustrated in Figure 1A, prior to immunization proliferative responses were not detected to any of the HER-2/neu peptides contained within the vaccine formulation. The stimulation index to p369-386 was 0.9, to p688-p703 was 0.9, and to p971-984 was 0.8. The patient demonstrated a proliferative response to tetanus toxoid (SI = 7.5). Following vaccination, 6 months after the initial immunization, a T-cell proliferative response was measured against 15-aa HER-2/neu peptide, p369-386 (Figure 1B). The stimulation index to p369-386 was 4.2, to p688-p703 was 1.4, and to p971-984 was 1.6. The tetanus toxoid response remained stable during the course of immunization and after the last vaccine was 4.9. The peptide, p369-386, encompassed within its sequence a defined HLA-A2 binding epitope, p369-377 [8, 15]. Twenty-one T-cell clones were isolated using this HLA-A2 peptide, representing a cloning efficiency of approximately 5%.

A polyclonal T-cell response could be elicited to p369-377, an HLA-A2 HER-2/neu epitope encompassed with the sequence of the helper epitope, after active immunization. The majority (19/21) of the clones were > 90% CD3⁺. Nineteen of 21 clones expressed the $\alpha\beta$ -TCR, and two clones expressed $\gamma\delta$ -TCR (clones IDI and 3F7). Representative histograms of TCR staining for 2 $\alpha\beta$ -TCR (example clones 2F10 and 2G2) and the 2 $\gamma\delta$ -TCR T-cell clones are depicted in Figure 2. Although the majority of the monoclonal populations expressed CD8 (16/21), some clones expressed CD4 (5/21) despite being cloned using the HLA-A2 peptide, p369-377. Representative flow cytometry dot plots from 4 $\alpha\beta$ -TCR clones, 2F10 (CD8⁺), 2G2 (CD8⁺), 2A2 (CD4⁺), and 3G9 (CD4⁺) stained with anti-CD8 and anti-CD4, are illustrated in Figure 3. Representative clones from each phenotypic group both $\alpha\beta$ or $\gamma\delta$ were chosen for evaluation in a more detailed fashion.

HER-2/neu peptide-specific $\alpha\beta$ -TCR clones secrete IFN- γ in response to antigen. The $\alpha\beta$ -TCR clones were examined for peptide-specific release of IFN- γ in an ELISPOT assay (Figure 4). Representative $\alpha\beta$ -TCR, CD8⁺ T cells were tested against p369-377, an irrelevant HER-2/neu 9 amino acid peptide, or no antigen. The number of spots detected in wells containing p369-377 was 137 ± 17 (mean \pm standard error of mean [SEM]), 177 ± 22 , and 102 ± 17 , for the clones, 3H8 (CD8), 2G2 (CD8), and 2F6 (CD8) respectively. These means were significantly greater than the mean number

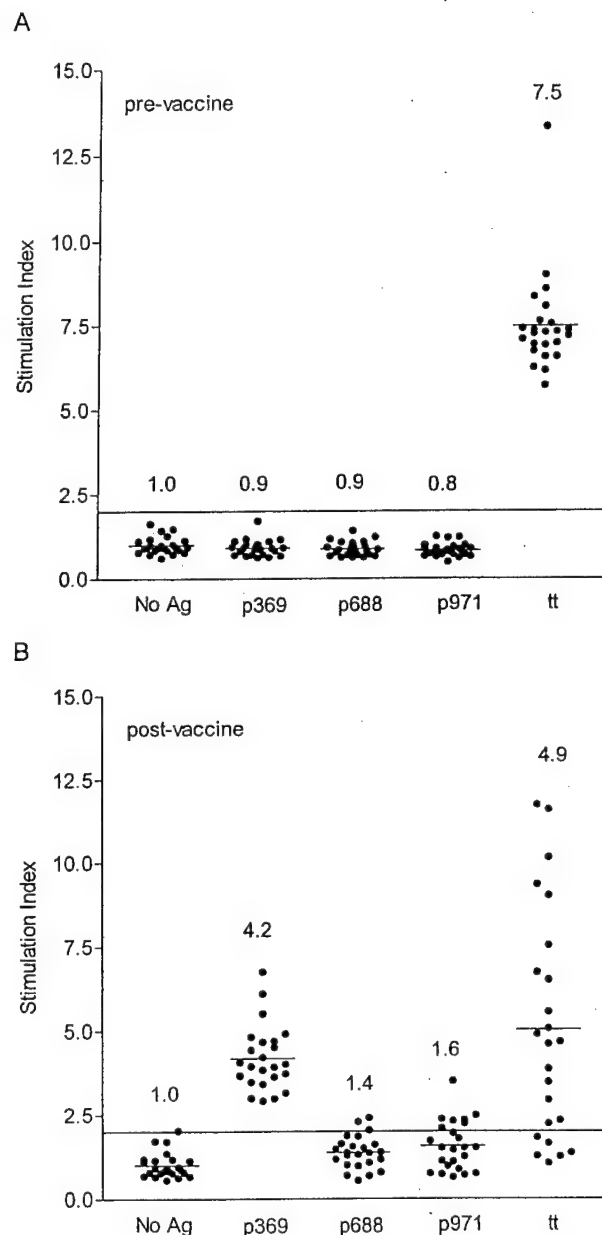


FIGURE 1 Immunity to HER-2/neu peptide, p369-386 is generated as a result of vaccination with helper peptides in a human leukocyte antigen (HLA-A2⁺) ovarian cancer patient. Depicted are the proliferation responses to media alone, the vaccine peptides, p369-386, p689-703, and p971-984. Tetanus toxoid (tt) was added as a positive control. Data from peripheral blood mononuclear cells drawn at the time of the first visit (Panel A) and the last immunization (Panel B) are illustrated. The long horizontal line spanning the width of the graph depicts average CPM of the no antigen wells + 3 standard deviation. The short horizontal lines within the data symbols represent the mean of the 24 replicates for each condition. The values above each data set are the calculated stimulation index.

Diversity of the T-cell Repertoire

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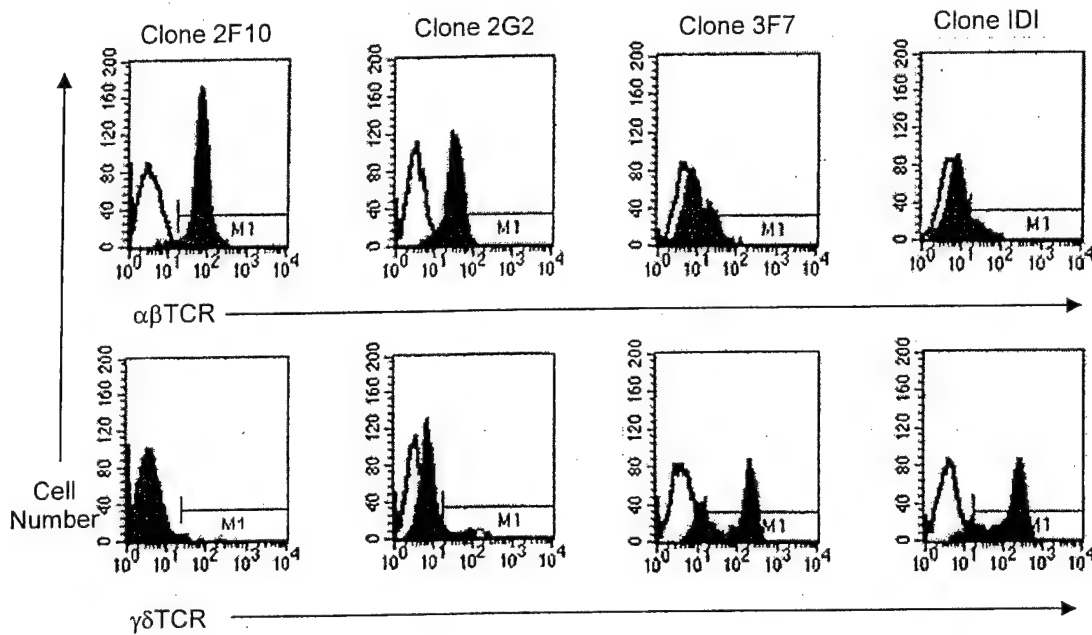


FIGURE 2 Both $\alpha\beta$ T-cell reponse ($\alpha\beta$ -TCR) and $\gamma\delta$ -TCR clones were isolated following HER-2/neu peptide vaccination. Illustration is a representative flow cytometry analysis, staining for either $\alpha\beta$ -TCR (top row) or $\gamma\delta$ -TCR (bottom row) of select T-cell clones. The results are representative of two independent experiments yielding similar results. The fluorescence intensity is plotted on the x axis and the cell counts are plotted on the y axis.

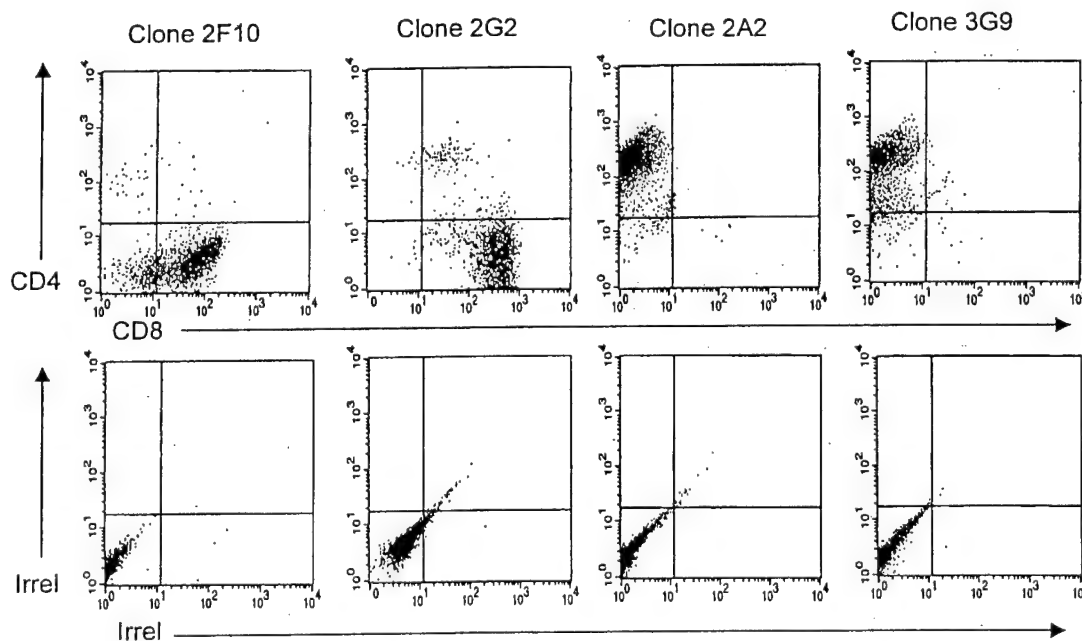


FIGURE 3 Both $CD4^+$ and $CD8^+$ clones were isolated following HER-2/neu peptide vaccination. Illustration is the representative flow cytometry data of p369-377-specific $\alpha\beta$ T-cell reponse ($\alpha\beta$ -TCR) T-cell clones dual-stained for both CD4 and CD8 (top row). Background irrelevant staining (FITC-conjugated mouse IgG) for the same clones are illustrated in the bottom row. The results are representative of two independent experiments yielding similar results.

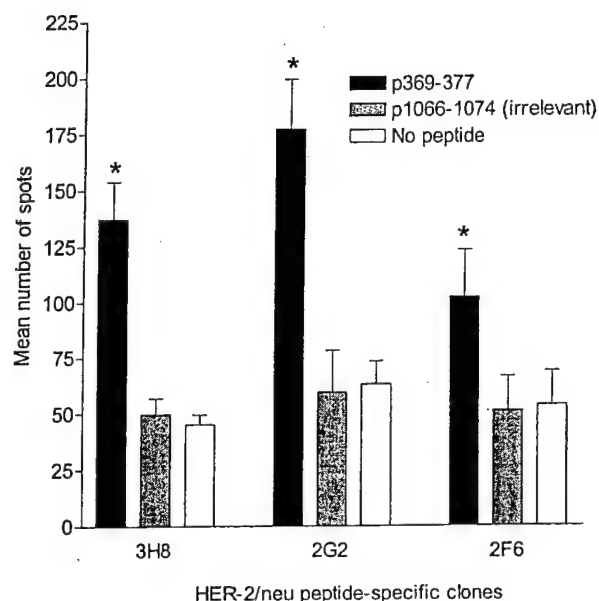


FIGURE 4 HER-2/neu peptide-specific $\alpha\beta$ -TCR clones secrete IFN- γ in response to antigen. ELISpot data from $\alpha\beta$ T-cell response ($\alpha\beta$ -TCR) clones 3H8, 2G2, and 2F6 are depicted presented as the mean (\pm SEM; * = $p < 0.05$) number of spots calculated from quadruplicate determinations for clones stimulated with p369-377 (black bars), p1066-1074 (gray bars), or no peptide (white bars).

of spots detected in wells containing either no antigen (3H8, 45 ± 4 , $p = 0.003$; 2G2, 63 ± 10 , $p = 0.002$; 2F6, 54 ± 15 , $p = 0.02$) or irrelevant peptide (3H8, 50 ± 7 , $p = 0.03$; 2G2, 59 ± 19 , $p = 0.03$; 2F6, 51 ± 15 , $p = 0.05$). These clones also all released IFN- γ into the supernatant as assessed by ELISA (data not shown).

HER-2/neu peptide-specific $\alpha\beta$ -TCR clones lyse HLA-A2⁺, HER-2/neu⁺ tumor cells. The clones were examined for lysis of peptide-loaded autologous BLCL or HER-2/neu-expressing tumor cell lines. As an example, two CD3⁺CD8⁺CD4⁺ clones (2G2 and 2G7) and one CD3⁺CD8⁺CD4⁺ (3G9) are revealed in Figure 5. The clones examined all demonstrated detectable lysis of p369-377-loaded BLCL and HLA-A2⁺ HER-2/neu-expressing tumor cells. At an effector:target (E:T) ratio of 40:1, the percent of specific lysis was 9%, 12%, and 13% for clones 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C), respectively against p369-377-loaded autologous BLCL. Responses against non peptide-loaded BLCL were < 4% for all clones tested. Against the HLA-A2⁺, HER-2/neu-expressing SKOV3 tumor cell line, the percent of specific lysis at 40:1 E:T was 7%, 7%, and 6% for 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C), respectively. Lysis of parental, HER-2-expressing SKOV3 tumor cells was not detectable for any of the tested clones.

The $\gamma\delta$ -TCR clones secrete IFN- γ and lyse HLA-A2⁺, HER-2/neu-expressing tumor cells. The $\gamma\delta$ -TCR clones were examined for expression of CD4, CD8 (Figure 6). Clone 3F7 was predominantly CD8⁺. In contrast, clone 1D1 displayed heterogeneous but consistent expression of CD4 and CD8. As assessed by flow cytometry expression of the $\gamma\delta$ -TCR was observed in 90% of cells (Figure 2). The remaining 10% expressed neither CD3 (not shown) nor $\gamma\delta$ -TCR suggesting nonspecific particulate debris. Approximately 50% of 1D1 expressed CD8, 10% expressed CD4, and the remaining 40% did not express either CD8 or CD4 (Figure 6). Peptide-specific release of IFN- γ against p369-377, an irrelevant HLA-A2 HER-2/neu peptide p1066-1074, or no antigen was examined in an ELISpot assay (Figure 7). The mean number of spots detected in wells containing p369-377 was $57 (\pm 7, \text{SEM})$ and $6 (\pm 1, \text{SEM})$ for 1D1 and 3F7 respectively. The mean number of spots, in the presence of p369-377, for 1D1 was significantly higher than the mean number of spots detected in wells containing either no antigen (19 ± 4 , SEM, $p = 0.015$) or irrelevant peptide (26 ± 8 , SEM, $p = 0.03$). Clone 3F7 did not demonstrate significantly elevated IFN- γ release in response to peptide.

The $\gamma\delta$ -TCR clones were also examined for lysis of HER-2/neu-expressing cells lines (Figure 8). Both clones displayed lysis of the HLA-A2⁺, HER-2/neu-expressing tumor cell line SKOV3-A2. At an E:T ratio of 40:1, the percent of specific lysis was 25% and 20% for 1D1 (Panel A) and 3F7 (Panel B), respectively. Lysis of parental HER-2-expressing SKOV3 tumor cells was < 1% for both clones at all E:T ratios examined.

CONCLUSIONS

We immunized patients with T-helper epitopes derived from HER-2/neu, each of which encompassed known HLA class I motifs to generate HER-2/neu-specific CTL in cancer patients [8]. Clinical vaccination with longer peptides encompassing HLA class I binding motifs allowed exogenous uptake of helper epitopes within the HLA class II processing pathway. Once internalized and processed, peptides are available to the HLA-class I processing pathway for presentation [16]. Thus, the subsequent stimulation of HLA class I restricted T cells represents an immune response that likely required internal processing and presentation of antigen rather than exogenous peptide loading. We cloned T cells from a patient successfully immunized with a T-helper peptide (p369-386) derived from HER-2/neu to evaluate the repertoire of the HLA class I specific peptide (p369-377) encompassed within the longer epitope. The studies described here demonstrate substantial diversity of the vaccinated repertoire, which consisted not only of CD4⁺ and CD8⁺.

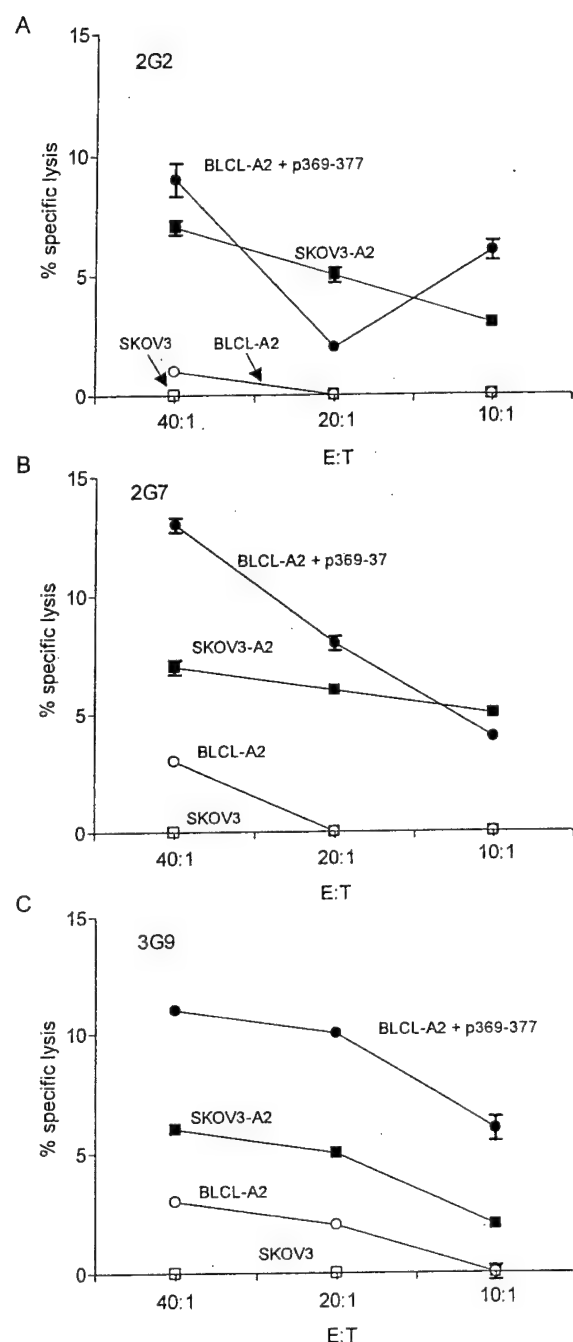


FIGURE 5 HER-2/neu peptide-specific $\alpha\beta$ T-cell response ($\alpha\beta$ -TCR) clones lyse human leukocyte antigen A2 (HLA-A2⁺), HER2/neu-expressing tumor cells. Cytolytic activity data from $\alpha\beta$ -TCR clones 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C) are depicted against BLCL-A2 alone (open circles), p369-377-loaded BLCL-A2 (closed circles), or the HER-2/neu-overexpressing tumor cells SKOV3 (open squares) and SKOV3-A2 (closed squares). The data are presented as the mean (\pm SEM) of triplicate determinations at each of three E:T ratios, 40:1, 20:1, and 10:1. The absence of a standard error bar indicates a SEM of $< 0.5\%$ specific lysis.

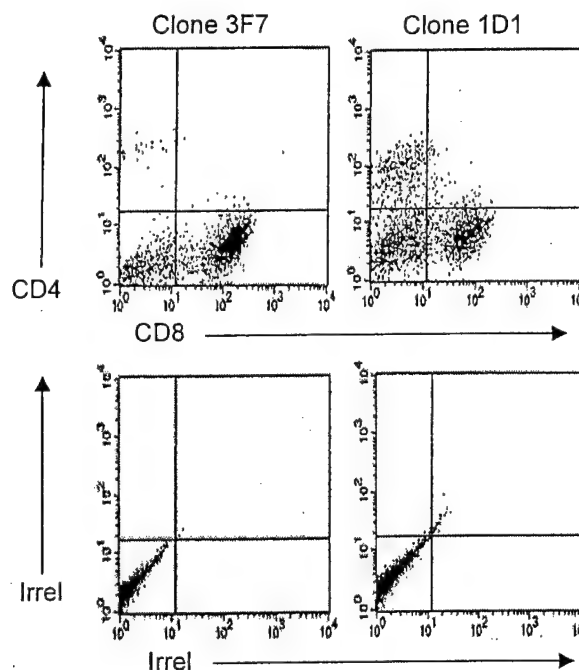


FIGURE 6 The $\gamma\delta$ T-cell response ($\gamma\delta$ -TCR) T-cell clones could be isolated following HER-2/neu vaccination. This illustrates the flow cytometry data of p369-377-specific $\gamma\delta$ -TCR T-cell clones dual-stained for both CD4 and CD8 (top row). Background irrelevant staining (FITC-conjugated mouse IgG) for the same clones are depicted in the bottom row. The results are representative of two independent experiments yielding similar results.

T cells, but also $\alpha\beta$ -TCR and $\gamma\delta$ -TCR T cells specific for HLA class I, HER-2/neu peptide antigen.

As expected, the majority of peptide-specific clones isolated were CD8⁺; however, nearly 25% of the clones identified were CD4⁺. A representative clone of the CD4⁺ T-cell population could lyse both peptide-loaded BLCL as well as HER-2/neu overexpressing HLA-A2 tumor cells. The lysis observed by CD4⁺ T-cell clones was weak and of similar magnitude to the lysis observed by the CD8⁺ T-cell clones, a finding that is consistent with previous studies of HER-2/neu-specific T-cell lines or clones [4, 10, 15, 17]. Isolation of T cells with weak lytic activity is likely due to loss of higher affinity T cells against self antigens by tolerizing mechanisms [18]. HLA class II restricted cytolytic CD4⁺ T cells have been defined and have been described to play a role in the pathogenesis of autoimmune disease via responses to self antigens, such as myelin [19]. Reports of HLA class I restricted CD4⁺ T cells are rare. There have been only a few reports of cytolytic CD4⁺ T cells responding to HLA class I peptides in melanoma [17, 20]. One study was a report of a HLA-B57-restricted CD4⁺ cytolytic clone, which could lyse autologous melanoma and allogeneic

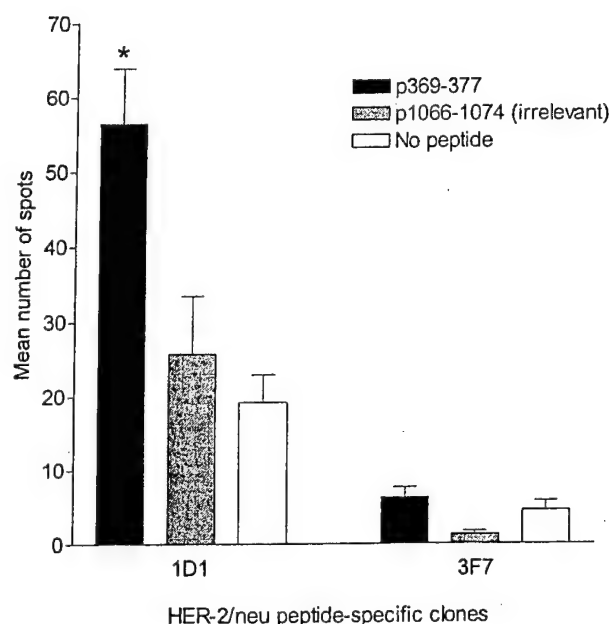


FIGURE 7 One of the CD8⁺ $\gamma\delta$ T-cell response ($\gamma\delta$ -TCR) clones, 1D1 secretes IFN- γ in response to antigen. ELISpot data from $\gamma\delta$ -TCR clones 1D1 and 3F7 are depicted and presented as the mean (\pm SEM; * = $p < 0.05$) number of spots calculated from quadruplicate determinations for clones stimulated with p369-377 (black bars), p1066-1074 (gray bars), or no peptide (white bars).

melanoma lines that were matched for HLA-B57 [16]. Nishimura and colleagues [17] performed a more extensive analysis of a CD4⁺ T-cell line derived from tumor infiltrating lymphocytes derived from a melanoma patient. The CD4⁺ T cells were specific for a tyrosinase peptide, p368-376, secreted cytokines of a Th1 phenotype and were weakly cytolytic. Furthermore, although the cell line demonstrated high CD4 expression, CD4 apparently was not involved in antigen-specific signaling through the TCR. The authors concluded that the HLA class I restricted CD4⁺ T cell was most likely a rare event with unknown functional significance. Data presented here suggests the HLA class I restricted CD4⁺ T cells may be present in number greater than previously thought. Whether the functional importance of these cells lay in direct killing or regulating the immune response through cytokine secretion is currently unknown.

Most analyses of the T-cell repertoire generated in response to dominant HLA class I epitopes have focused exclusively $\alpha\beta$ -TCR⁺, CD3⁺, CD8⁺ T cells. Specifically, investigations have analyzed the V β usage and the CDR3 size of the $\alpha\beta$ -TCR of viral peptide-specific T cells [21, 22]. In general, findings from viral studies suggest that the repertoire to dominant viral epitopes is

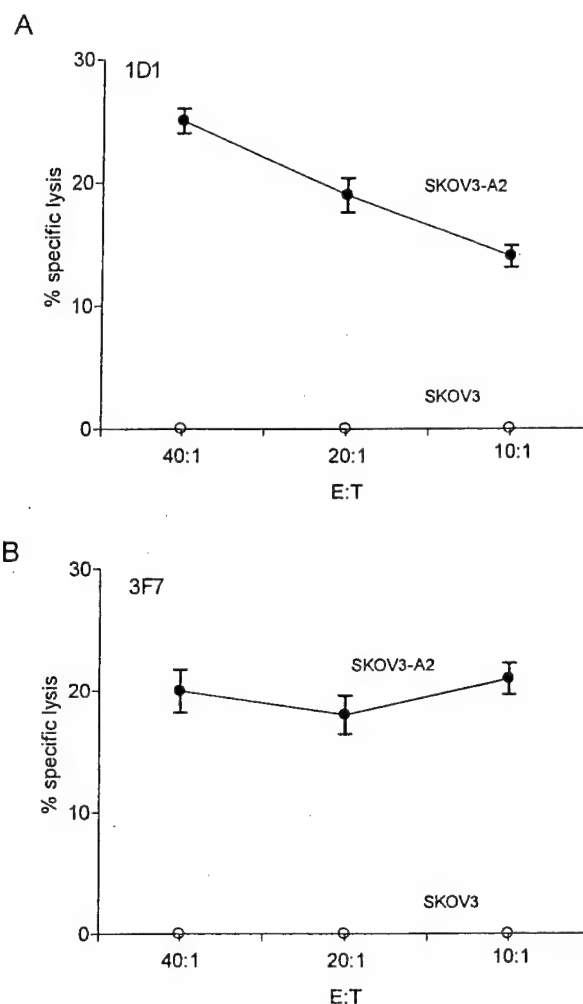


FIGURE 8 The CD8'2b $\gamma\delta$ T-cell response ($\gamma\delta$ -TCR) clones displayed human leukocyte antigen A2 (HLA-A2) restricted, HER2⁺ tumor cell lysis. Cytolytic activity data from $\gamma\delta$ -TCR clones 1D1 (Panel A) and 3F7 (Panel B) are depicted against the HER-2/neu overexpressing tumor cells SKOV3 (open circles) and SKOV3-A2 (closed circles). The data are presented as the mean (\pm SEM) of triplicate determinations at each of three E:T ratios, 40:1, 20:1, and 10:1.

tightly restricted in terms of TCR usage. Recently, however, Dietrich and colleagues [23] examined the clonality of the T-cell repertoire to an HLA-A2 restricted immunodominant epitope of the melanoma-associated antigen, Melan-A. They observed, by analyzing the V β segment and CDR3 lengths, that the natural repertoire to this self peptide was considerably broader than what had been previously observed in viral peptide systems, suggesting that the $\alpha\beta$ -TCR usage is not as narrowly restricted in response to tumors as it is in viral infections. As expected, in the present study, the predominant TCR was the $\alpha\beta$ -TCR. However, 2 of 21 clones (10%) expressed

$\gamma\delta$ -TCR. $\gamma\delta$ -TCR T cells are involved in a wide range of immune responses to infectious and non-infectious diseases, including malaria, mycobacterial infections, cancers, and autoimmune disorders, such as multiple sclerosis [24]. The $\gamma\delta$ -TCR T-cell clones have been isolated from tumor-infiltrating lymphocytes derived from tumors, such as dysgerminoma [25], seminoma [25], renal carcinoma [26], lung [27], colorectal [28], and melanoma [29]. Tumor-associated $\gamma\delta$ -TCR T cells that have been described in colorectal cancers have a CD8⁺, cytolytic phenotype similar to those described in the present study [27]. In the present study, $\gamma\delta$ -TCR T-cell clone 1D1, displayed heterogeneous expression of CD4 or CD8. In general, circulating $\gamma\delta$ -TCR T cells do not express CD4 or CD8, which is distinct from $\alpha\beta$ -TCR T cells in which either CD4 or CD8 expression occurs during early thymocyte ontogeny [30]. Little is known about the regulation of expression of CD4 and CD8 in $\gamma\delta$ -TCR T cells, but since the majority of circulating $\gamma\delta$ -TCR T cells are CD4⁻/CD8⁻, it is thought that some coreceptor upregulation may be due to environmental maturation [24, 31]. Therefore, it is possible that the differential expression of CD8 and CD4 in our clonal population was induced by *in vitro* maturation leading that could have led to clonal divergence [32].

In autoimmune diseases, pathology has been attributed to infiltrating $\gamma\delta$ -TCR T cells. For example, antibody depletion of $\gamma\delta$ -TCR reduces demyelination and inflammation in experimental murine multiple sclerosis [33]. It is unknown if autoreactive $\gamma\delta$ -TCR T cells respond secondarily to damaged and stressed tissue [34] or if they initiate autoimmunity directly. One hypothesis [35] is that, given the broad range of regulation by multiple mechanisms of antigen presentation and natural localization to epithelial tissue, $\gamma\delta$ -TCR T cells are sentinels for the immune system and are capable of alerting the immune system to the presence of danger (e.g., infection, tumors, etc.).

Immunization against tumor antigens in cancer patients with peptide-based vaccines has been best studied in patients with existing disease burden and clinical responses have occurred only infrequently [36, 37]. We hypothesize that vaccines may be better suited for the prevention of relapse following conventional therapies [8]. Nevertheless, characterization of the T-cell response to tumor antigen-specific peptide vaccines is necessary for optimizing design and improving clinical outcome. The results presented here demonstrate that the T-cell population specific for the HER-2/neu peptide, p369-377 elicited after active immunization is polyclonal both at the level of the TCR and the T-cell subsets stimulated. These data reflect the distinct differences between the highly monoclonal immunodominant responses directed against foreign infectious disease antigens and the diverse

polyclonal response against self tumor antigens. Many of the elements in the immune response described in this report are important in the autoimmunity (i.e., CD4⁺ cytolytic T cells and $\gamma\delta$ TCR T cells). Perhaps functional immunity directed against specific self antigens mimics the pathogenic pathways of autoimmune disease closer than anticipated.

ACKNOWLEDGMENTS

We express our gratitude to the ovarian cancer patient who participated in this study. This work is supported for KLK by a fellowship from the Department of Defense Breast Cancer Program; and for MLD by grants from the NIH, NCI (R01 CA75163), and the Department of Defense Breast Cancer Program. Patient care was conducted through the Clinical Research Center Facility at the University of Washington, which is supported through NIH grant MO1-RR-00037. We are grateful for the expert nursing care and protocol coordination by Lynne Fitzsimmons, R.N., O.C.N., and Donna Davis, B.S.N.

REFERENCES

1. Eisenbach L, Bar-Haim E, El-Shami K: Antitumor vaccination using peptide based vaccines. *Immunol Lett* 74:27, 2000.
2. Disis ML, Gralow JR, Bernhard H, Hand SL, Rubin WD, Cheever MA: Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self-protein. *J Immunol* 156:3151, 1996.
3. Hunziker IP, Zurbriggen R, Glueck R, Engler OB, Reichen J, Dai WJ, Pichler WJ, Cerny A: Perspectives: towards a peptide-based vaccine against hepatitis C virus. *Mol Immunol* 38:475, 2000.
4. Zaks TZ, Rosenberg SA: Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. *Cancer Res* 58:4902, 1998.
5. Chen W, Norbury CC, Cho Y, Yewdell JW, Bennink JR: Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. *J Exp Med* 193:1319, 2001.
6. Irvine KR, McCabe BJ, Rosenberg SA, Restifo NP: Synthetic oligonucleotide expressed by a recombinant vaccinia virus elicits therapeutic CTL. *J Immunol* 154:4651, 1995.
7. Bonini C, Lee SP, Riddell SR, Greenberg PD: Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4(+) and CD8(+) T cells. *J Immunol* 166:5250, 2001.
8. Knutson KL, Schiffman K, Disis ML: Immunization with a HER-2/neu helper peptide vaccine generates of HER-2/neu CD8 T cell immunity in cancer patients. *J Clin Invest* 107:477, 2001.

9. Fisk B, Hudson JM, Kavanagh J, Wharton JT, Murray JL, Ioannides CG, Kudelka AP: Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. *Anticancer Res* 17:45, 1997.
10. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci U S A* 92:432, 1995.
11. Ioannides CG, Fisk B, Fan D, Biddison WE, Wharton JT, O'Brian CA: Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. *Cell Immunol* 151:225, 1993.
12. Disis ML, Grabstein KH, Sleath PR, Cheever MA: Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin Cancer Res* 5:1289, 1999.
13. Disis ML, Bernhard H, Gralow JR, Hand SL, Emery SR, Calenoff E, Cheever MA: Immunity to the HER-2/neu oncogenic protein. *Ciba Found Symp* 187:198, 1994.
14. Reece JC, Geysen HM, Rodda SJ: Mapping the major human T helper epitopes of tetanus toxin. The emerging picture. *J Immunol* 151:6175, 1993.
15. Fisk B, Blevins TL, Wharton JT, Ioannides CG: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* 181:2109, 1995.
16. Rock KL, Clark K: Analysis of the role of MHC class II presentation in the stimulation of cytotoxic T lymphocytes by antigens targeted into the exogenous antigen-MHC class I presentation pathway. *J Immunol* 156:3721, 1996.
17. Nishimura MI, Avichezer D, Custer MC, Lee CS, Chen C, Parkhurst MR, Diamond RA, Robbins PF, Schwartzentruber DJ, Rosenberg SA: MHC class I-restricted recognition of a melanoma antigen by a human CD4⁺ tumor-infiltrating lymphocyte. *Cancer Res* 59:6230, 1999.
18. Slansky JE, Rattis FM, Boyd LF, Fahmy T, Jaffee EM, Schneck JP, Margulies DH, Pardoll DM: Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity* 13:529, 2000.
19. Martino G, Hartung HP: Immunopathogenesis of multiple sclerosis: the role of T cells. *Curr Opin Neurol* 12:309, 1999.
20. Somasundaram R, Robbins P, Moonka D, Loh E, Marincola F, Patel A, Guerry D, Herlyn D: CD4(+), HLA class I-restricted, cytolytic T-lymphocyte clone against primary malignant melanoma cells. *Int J Cancer* 85:253, 2000.
21. Lim DG, Bieganski BK, Freeman GJ, Hafler DA: Examination of CD8⁺ T cell function in humans using MHC class I tetramers: similar cytotoxicity but variable proliferation and cytokine production among different clonal CD8⁺ T cells specific to a single viral epitope. *J Immunol* 165:6214, 2000.
22. Chen ZW, Li Y, Zeng X, Kuroda MJ, Schmitz JE, Shen Y, Lai X, Shen L, Lervin NL: The TCR repertoire of an immunodominant CD8(+) T lymphocyte population. *J Immunol* 166:4525, 2001.
23. Dietrich PY, Walker PR, Quiquerez AL, Perrin G, Dutoit V, Lienard D, Guillaume P, Cerottini JC, Romero P, Valmori D: Melanoma patients respond to a cytotoxic T lymphocyte-defined self-peptide with diverse and non-overlapping T-cell receptor repertoires. *Cancer Res* 61:2047, 2001.
24. Boismenu R, Havran WL: Gammadelta T cells in host defense and epithelial cell biology. *Clin Immunol Immunopathol* 86:121, 1998.
25. Zhao X, Wei YQ, Kariya Y, Teshigawara K, Uchida A: Accumulation of gamma/delta T cells in human dysgerminoma and seminoma: roles in autologous tumor killing and granuloma formation. *Immunol Invest* 24:607, 1995.
26. Choudhary A, Davodeau F, Moreau A, Peyrat MA, Bonneville M, Jotereau F: Selective lysis of autologous tumor cells by recurrent gamma delta tumor-infiltrating lymphocytes from renal carcinoma. *J Immunol* 154:3932, 1995.
27. Yu S, He W, Chen J, Zhang F, Ba D: Expansion and immunological study of human tumor infiltrating gamma/delta T lymphocytes in vitro. *Int Arch Allergy Immunol* 119:31, 1999.
28. Watanabe N, Hizuta A, Tanaka N, Orita K: Localization of T cell receptor (TCR)-gamma delta + T cells into human colorectal cancer: flow cytometric analysis of TCR-gamma delta expression in tumour-infiltrating lymphocytes. *Clin Exp Immunol* 102:167, 1995.
29. Bachelez H, Flageul B, Degos L, Boumsell L, Bensussan A: TCR gamma delta bearing T lymphocytes infiltrating human primary cutaneous melanomas. *J Invest Dermatol* 98:369, 1992.
30. Ellmeier W, Sawada S, Littman DR: The regulation of CD4 and CD8 coreceptor gene expression during T cell development. *Annu Rev Immunol* 17:523, 1999.
31. Kabelitz D, Wesch D, Hinz T: gamma delta T cells, their T cell receptor usage and role in human diseases. *Springer Semin Immunopathol* 21:55, 1999.
32. Goodman T, Lefrancois L: Expression of the gamma-delta T-cell receptor on intestinal CD8⁺ intraepithelial lymphocytes. *Nature* 333:855, 1988.
33. Rajan AJ, Gao YL, Raine CS, Brosnan CF: A pathogenic role for gamma delta T cells in relapsing-remitting experimental allergic encephalomyelitis in the SJL mouse. *J Immunol* 157:941, 1996.
34. Hayday A, Geng L: Gamma delta cells regulate autoimmunity. *Curr Opin Immunol* 9:884, 1997.
35. De Libero G: Tissue distribution, antigen specificity and effector functions of gamma delta T cells in human diseases. *Springer Semin Immunopathol* 22:219, 2000.
36. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Glad-

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haug I, Soreide O, Eriksen JA, Moller M, Baksaas I, Lothe RA, Saeterdal I, Gaudernack G: Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: clinical and immunological responses in patients with pancreatic adenocarcinoma. *Int J Cancer* 92:441, 2001.

37. Lee KH, Wang E, Nielsen MB, Wunderlich J, Migueles S, Connors M, Steinberg SM, Rosenberg SA, Marincola FM: Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. *J Immunol* 163:6292, 1999.

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APPENDIX 3

Running Title: IL-12 activates tumor antigen-specific helper T cells

IL-12 enhances the function and number of tumor antigen-specific Th1 lymphocytes during *ex vivo* expansion.

Keith L. Knutson and Mary L. Disis

Division of Oncology, University of Washington
Seattle, WA 98195

Corresponding Author and Address for Reprints: Dr. K.L. Knutson, Division of Oncology, University of Washington, 1959 NE Pacific Street, HSB BB1321, Box 356527, Seattle, WA 98195-6527, Telephone: 206-221-5417, Fax: 206-685-3128, Email: kknutson@u.washington.edu.

Abstract

CD4⁺ T cells are essential for the immune response against cancer. Vaccination against cancer will likely only be effective at preventing growth of micrometastatic disease while adoptive T cells therapy will be better suited for eradication of bulky pre-existing disease. Problems with the translation of adoptive T cell therapy include lack of CD4⁺ T cell help, low precursor frequency of antigen-specific T cells and lack of effective *ex vivo* expansion techniques. In this study, we focused on improving *ex vivo* expansion of T helper cells. The effects of IL-12, along with IL-2, on the *ex vivo* generation of HER-2/neu antigen-specific T cells were examined. Patients were immunized with a peptide-based vaccine that contained a helper epitope, p776-790, derived from the intracellular domain of HER-2/neu. While immunity to p776-790 could be readily measured in short-term cultures, cell line generation by multiple *in vitro* stimulation with peptide and IL-2 as the only added cytokine resulted in loss of activity. The inclusion of IL-12, along with IL-2, restored antigen-specific responsiveness in a dose dependent fashion. The resulting p776-790-specific T cells responded readily to antigen by proliferating and producing type I cytokines (IFN- γ and TNF- α). The increased proliferative response of the cultures was due in part to an increase in the number of HER-2/neu-specific T cells. IL-12 inclusion also resulted in the decrease of non-specific cellular proliferation. These results suggest that IL-12 is an important cytokine for *ex vivo* recovery and maintenance of antigen-specific CD4⁺ T lymphocytes that would otherwise be lost by using IL-2 alone in combination with antigen. Furthermore, these results have important implications for *ex vivo* expansion of CD4⁺ T cell for use in anti-tumor adoptive immunotherapy.

Keywords: IL-12, T lymphocyte, cytokine, tumor antigen, peptide.

Introduction

Studies of human adoptive T cell therapy in both animal and humans demonstrate that CD4⁺ T cell help is required to sustain and increase the effectiveness of CD8⁺ T cells (1, 2). Thus, the elucidation of improved culture conditions for the *ex vivo* expansion of CD4⁺ T cells is an important issue (3). Currently used *ex vivo* culture conditions are often inadequate for expansion of CD4⁺ T cells. The use of IL-2 alone during cell line generation with antigen can result in loss of antigen specificity, particularly when antigen-specific T cell precursors are low.

With the discovery and characterization of novel cytokines it has become possible to test other cytokines used in conjunction with IL-2 to improve preferential expansion of bulk cultured tumor antigen-specific CD4⁺ T lymphocytes *ex vivo* without the loss of functionality. However, many CD4⁺ T cell-active cytokines such as IL-7 and IL-16 do not preferentially activate antigen-specific CD4⁺ T cells but rather can lead to non-specific expansion of all lymphocytes, including NK cells, in bulk cultures. For example, while studies clearly demonstrate that IL-7 can promote the *ex vivo* expansion of tumor-specific cytolytic CD8⁺ T cells, the limited studies of the effects of IL-7 on the culture of CD4⁺ T cells suggests that inclusion of IL-7 with IL-2 results in increased non-specific proliferative activity (4). Indeed, a suggested role for IL-7 is maintaining the naïve CD4⁺ T cell pool through the non-specific antigen-independent proliferation. (5). IL-16 promotes the proliferation of CD4⁺ T cells and can result in significantly enhanced CD4⁺ T cell yield following culture in the presence of IL-2. But, like IL-7 however, IL-16 acts non-specifically on all cells expressing CD4⁺ (6).

Another cytokine that has been extensively characterized over the past decade is IL-12, a cytokine that is uniquely different from either IL-7 or IL-16 in that it only acts on activated T cells (7). While the effects of IL-12 are well characterized on the *ex vivo* expansion of antigen-specific CD8⁺ T

cells (8, 9) little is known about the role of IL-12 during the *ex vivo* expansion of CD4⁺ T cells, particularly T cells specific for tumor antigens.

In the present study, the *in vitro* effects of IL-12, along with IL-2, on CD4⁺ T cell line growth, phenotype, and antigen-specific function were evaluated. HER-2/neu peptide, p776-790 (10) was used as a model to study the role of IL-12 during *in vitro* cell growth of antigen-specific CD4⁺ T cells.

Results

Generation of immunity to HER-2/neu helper peptide, p776-790, in patients with HER-2/neu-overexpressing tumors. Patients 1276 and 6622 were vaccinated with a HER-2/neu peptide-based vaccine consisting of helper peptides including p776 (11). The patients had no detectable preexisting immunologic response to the p776 or the HER-2/neu ICD. However, after the immunizations were complete, patients had detectable p776-specific proliferation responses (Fig. 1). Both patients also developed ICD-specific protein proliferation responses. The patients did not develop responses to another HER-2/neu-derived peptide, p42, which was not contained in the vaccine preparation. PBMC derived from each patient donor were used to assess the effects of IL-12 on antigen-specific T cell line expansion and phenotype using p776 as a model antigen.

The addition of IL-12 to IL-2 increases antigen-specific clonal proliferation after IVS with HER-2/neu helper peptide. Proliferation was assessed following IVS of patient PBMC with p776, IL-2 and varying (0, 0.01, 0.1, 1, and 10 ng/ml) concentrations of IL-12. As shown in Figure 2, those cells expanded in culture in the presence of IL-2 alone, i.e. in the absence of IL-12, did not demonstrate a proliferative response to p776. The addition of IL-12, during IVS, resulted in augmentation of antigen-specific proliferation in a dose-dependent manner. As an example, the SI of T cells carried through IVS in 10 ng/ml IL-12 was 16 in PBMC from patient 1276 (Fig. 2A) and 9 in PBMC from patient 6622 (Fig. 2B). The proliferative responses were specific for the p776, as there was no proliferative response to the irrelevant HER-2/neu peptide, p42 (data not shown). The inclusion of IL-12 along with IL-2 during IVS substantially reduced non-specific cellular proliferation (Figs 2C and 2D). The reduction in non-specific cellular proliferation was similar at all concentrations of IL-12 used and was not dose-dependent within the range of IL-12 concentrations used.

The addition of IL-12 to IL-2 increases the CD4⁺ T cell precursor frequencies after IVS with HER-2/neu helper peptide. The precursor frequency was evaluated following IVS of patient PBMC with p776 and IL-2 in the absence or presence of IL-12 (Fig. 3). Following IVS with peptide and IL-2 alone, the p776-specific and p42-specific precursor frequencies in PBMC from patient 1276 were 91 ± 22 /million cells and 0/million cells, respectively (Fig. 3A). The addition of IL-12 during IVS resulted in an increase of p776-specific T cells to 1003 ± 50 /million cells. In the absence of IL-12 the precursor frequencies to p776 and p42 in PBMC from patient 6622 were not detectable following IVS with p776 and IL-2 alone (Fig. 3B). The inclusion of IL-12 resulted in increased p776-specific precursors to 272 ± 24 /million cells. T cell precursors specific for p42 were not elevated in PBMC from either patient as a result of inclusion of IL-12 during IVS (Figs. 3A and 3B).

The addition of IL-12 to IL-2 increases antigen-specific TNF- α release by T cells after IVS with HER-2/neu helper peptide. Figure 4 demonstrates that IL-12 in culture can enhance tumor antigen-induced TNF- α release compared to cultures incubated in IL-2 alone. For patient 1276, the TNF- α yield from T cell lines established in p776 and IL-2 alone was 63 ± 7 pg/ml and increased to 390 ± 33 pg/ml by the inclusion of IL-12 (Fig. 4A). For patient 6622, the TNF- α yield from T cell lines established in p776 and IL-2 alone was not detectable and was increased to 9.5 ± 3 pg/ml for cells incubated by the inclusion of IL-12 (Fig. 4B). Increased TNF- α release to the irrelevant HER-2/neu peptide, p42-15, was not observed in PBMC in either patient (Figs 4C and 4D).

Discussion

In general, active immunization of cancer patients with tumor antigen-specific vaccine does not result in regression or cure of existing malignancy. Most likely, the greatest utility of cancer vaccines will be in the prevention of cancer recurrence or even the initiation of disease. Infusion of competent antigen-specific T cells however, may result in eradication of cancer (12). Successful *ex vivo* expansion of tumor-specific T cells for therapeutic use in humans has been limited by the lack of defined tumor antigens which would allow expansion of antigen-specific T cells, the understanding of the *in vitro* expansion requirement of T cells which would allow the generation of maximal numbers while retaining optimal antigen-specific function, and the ability to develop methods for *ex vivo* culture which could expand polyclonal T cell lines, both CD4⁺ and CD8⁺.

HER-2/neu is a tumor antigen. Active immunization of cancer patients with HER-2/neu peptide vaccine can boost antigen-specific T cell precursor frequencies substantially *in vivo* (13). We hypothesized that active immunization of cancer patients followed by *ex vivo* expansion of T cells may offer the clinical potential of increasing cancer-specific T cells to numbers greater than what could be achieved by vaccination alone. We evaluated *ex vivo* expansion of T cells specific for HER-2/neu helper epitopes as a model using IL-2 and IL-12 to augment T cell number and function.

We focused on expanding a Th1 CD4⁺ T cell population. It is well known that the CD4⁺ T cell is critical in controlling the activation and persistence of the immune response against viral infections (1, 2). Recent evidence also suggests that CD4⁺ T cells are essential for anti-tumor immunity and for function and survival of tumor-specific CD8⁺ T cells (14). In murine tumor models, lymphocyte depletion studies reveal that both CD8⁺ and CD4⁺ T cells are required for therapeutic efficacy (15). In fact, CD4⁺ T cell-depleted mice showed faster tumor development than CD8⁺-depleted mice suggesting a dominant role for CD4⁺ T cells (16). The importance of CD4⁺ T cells in the evolution of

the tumor-specific immune response is underscored by recent studies demonstrating that infusion of tumor antigen-specific CD4⁺ T cells can initiate a *de novo* tumor antigen-specific CTL response (17). CD4⁺ T cells can also modify the immune microenvironment by producing cytokines that attract DC, macrophages, and eosinophils to promote an inflammatory environment (18).

We examined the utility of IL-12, in combination with IL-2, on the expansion and function of T cell lines specific for an immunodominant helper peptide of HER-2/neu against which patients were actively immunized (13). IL-12 has been well defined for *ex vivo* expansion of viral-specific CD8⁺ T cells. For example, the cytolytic activity of HIV-specific T lymphocytes is greatly enhanced by including IL-12 along with IL-2 during *ex vivo* expansion of T cells with HIV-derived antigens (19). Less is known about a role for *ex vivo* expansion of CD4⁺ T cell lines using IL-12 and specifically the generation of Th1 T cell lines reactive against human tumor antigens. By using a minimal MHC class II-restricted helper peptide directly as the antigen, we were able to eliminate any variability associated with using whole protein or cellular antigen such as activation of multiple T cell subsets including CD4⁺ and CD8⁺ T cells, the presentation of subdominant epitopes, or even the presence of contaminants. Experiments demonstrate that inclusion of IL-12 along with IL-2 during the *ex vivo* expansion of human antigen-specific CD4⁺ T cells resulted in a significant increase in the blastogenic response to a subsequent rechallenge with antigen, the number of antigen-specific T cells, and augmented function of Th1 phenotype lymphocytes.

We observed that IL-12 increases the proliferation response and the numbers of tumor antigen-specific CD4⁺ T cells. CD4⁺ T cell lines specific for environmental allergens have increased proliferative capacity following incubation with IL-12 and IL-2 (20). However, it is unclear from these previous studies if the effects of IL-12 are a direct result of increased antigen-specific T cell precursors or rather an increased proliferative capability of individual antigen-specific T cells. By

limiting dilution analysis, Uherova observed 1.5 to 2-fold increases in the numbers of total cells responding to Candida antigen or tetanus toxoid when IL-12 was added to PBMC in the absence of IL-2 (21). Nevertheless, these results suggested that IL-12 might result in increased T cell precursors rather than increasing the proliferation rate of the individual CD4⁺ T cells. Recent findings indicate that IL-12 does not directly influence the rate of proliferation of cloned human CD4⁺ T cells (22). Our findings demonstrate that IL-12 can result in increased antigen-specific T cell precursors which may be responsible, in part, for the increased proliferation response of the antigen-specific CD4⁺ T cell lines.

In the present study, the addition of IL-12 also increased the function of the antigen-specific helper T cell lines as observed by the increased production of both IFN- γ and TNF- α . This is in agreement with previous findings where IL-12 is consistently found to preferentially induce a Th1 polarization in CD4⁺ T cells (23). IL-12 is also known to enhance cytokine production in individual clonal T cells (23). Th1 responses are important in many immunotherapeutic settings, ranging from the treatment of intracellular bacterial pathogens, viruses, and tumors. A Th1 immune response supports the polyclonal expansion and persistence of CD8⁺ T cells that directly kill infected cells and tumor cells. Furthermore, CD4⁺ T cells can kill tumor cells secondarily through TNF- α production since the cytokine can directly induce apoptosis of tumor cells (24).

In summary, the results presented here demonstrate that IL-12, when added to cell cultures during IVS with IL-2 and tumor antigen helper peptides promotes increased recovery of T cells specific for HER-2/neu helper peptide. T cell lines incubated with antigen, IL-2 and IL-12 show augmented function and secretion of Th1 type cytokines. Our findings could potentially be useful for translating adoptive T cell therapy to the clinic where increasing both numbers and function of polyclonal tumor antigen specific T cells may be important in mediating an anti-tumor response.

Materials and Methods

Materials: All peptides were >95% purity and manufactured by United Biochemical Inc. (Seattle, WA). HER-2/neu peptides used were p42-56 (p42), HLDMLRHLYQGCQVV (11) and p776-790 (p776), GVGSPYVSRLLGICL (10). Nitrocellulose-backed plates were purchased from Millipore Corp (Bedford, MA). Anti-IFN- γ monoclonal antibodies were purchased from Mabtech (Nacka, Sweden). IL-12 (batch # 4D18I002, 1.7×10^7 U/mg) was a generous gift from the Genetics Institute (Cambridge, MA). Anti-CD8, anti-CD4, anti- $\alpha\beta$ -TCR, anti-CD3, anti-TNF- α antibodies and purified recombinant human TNF- α were from Pharmingen (San Diego, CA), streptavidin-alkaline phosphatase and alkaline-phosphatase color detection reagents from Bio-Rad Laboratories (Hercules, CA), human AB⁺ serum from Valley Biomedical, Incorporated (Winchester, VA), and Immulon IV ELISA plates from Dynex Technologies (Chantilly, VA). TMB peroxidase substrate was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD) and streptavidin-horseradish peroxidase was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Source and Preparation of PBMC. Breast cancer patients participating in a Phase I trial of HER-2/neu peptide vaccines were leukapheresed at the completion of the trial after informed consent. PBMC derived from those leukaphereses were used in these studies (11). PBMC were isolated by density gradient centrifugation as previously described (11). Cells were aliquoted and cryopreserved in liquid nitrogen in freezing media (90% fetal bovine serum and 10% dimethylsulfoxide) at a cell density of $25\text{--}50 \times 10^6$ cells/ml.

In vitro stimulation (IVS)¹ of PBMC with peptide: In all experiments, except as indicated, PBMC were stimulated *in vitro* for 12 days in the presence of p776 peptide. For each experiment, $40\text{--}80 \times 10^6$ cryopreserved cells were rapidly thawed, washed 2 times in 10 mls of RPMI-1640 containing streptomycin, penicillin, and 10% human AB serum (complete medium), and resuspended at $1.0\text{--}2.0 \times$

10^6 cells/ml in the same medium and incubated in a humidified incubator at 37°C at 5% CO₂. Peptide was added on day 0 to a concentration of 10 µg/ml. Ten U/ml of IL-2 and 0.01-10 ng/ml of IL-12 were added on days 4 and 8 and the cells were further incubated for 4 days.

T-lymphocyte proliferation: Thymidine incorporation was measured as previously described (13, 25). The generation of immune responses by peptide vaccination were detected from PBMC samples and presented as the mean of 24 well replicates, each well containing 2.5×10^5 cells. The detection of proliferation following IVS is presented as the mean SI of 3-6 replicate wells each containing 7.5×10^4 cells. No cytokines (i.e. IL-2 and IL-12) were added during proliferation assessment. SI's of greater than 2 were considered a positive proliferation response as previously described (12).

Cytokine secretion: Following IVS, cells were restimulated with 10 µg/ml peptide and irradiated, autologous PBMC at 2.5×10^5 /well for 40 hours. No cytokines (i.e. IL-2 and IL-12) were added during restimulation. TNF-α content of the media was measured by ELISA. Immulon IV ELISA plates were coated with 50 µl of mouse anti-TNF-α monoclonal antibody at 2.5 µg/ml in carbonate buffer (15 mM Na₂CO₃, 35 mM NaH/CO₃, pH 8.0). Wells were blocked 2 hours at room temperature with 200 µl of PBS containing 1% BSA and 0.5% Tween-20. The plates were washed 4 times using PBS containing 0.1% Tween-20 (PBST). Cell culture supernatants were added at 100 µl/well and the plates were further incubated overnight at 4°C. The plates were washed 4 times with PBST. One hundred microliters PBST containing 10% goat serum and 0.5 µg/ml biotinylated anti-TNF-α antibody were added to wells and the plates were further incubated for 2 hours at room temperature followed by washing 4 times with PBST. The plates were subsequently incubated with 100 µl/well of PBS containing 1:3000 streptavidin-horseradish peroxidase, washed, and developed with 100 µl/well TMB peroxidase substrate. The developing reaction was terminated with 1N

hydrochloric acid and the OD was measured at 450 nm optical wavelength using a Molecular Devices (Sunnyvale, CA) kinetic microplate reader. Purified TNF- α was analyzed in parallel and the OD values of the samples were used to establish a standard curve (ng/ml).

Enzyme-linked immunosorbent spot (ELIspot): An ELIspot assay was used to determine precursor frequencies of peptide-specific T lymphocytes as previously described with some minor modifications (13). On day 1, T cells (3-6 replicates per condition) were plated into 96-well, anti-IFN- γ -coated nitrocellulose plates in 100 μ l media. The cells were stimulated with 100 μ l of media containing a 1:1 ratio of autologous, irradiated (3000 rads) PBMC with or without antigen (10 μ g/ml). The cells were further incubated for 20 hr at 37⁰C and detection of bound IFN- γ was performed as previously described (13). Data is presented as the mean precursor frequency/million cells (\pm sem) calculated from 6 (patient 1276) or 3 replicates (patient 6622).

Acknowledgements: This work was supported for KKK by a fellowship from the Department of Defense Breast Cancer Program and for MLD by NCI grants R01CA85374 and K24CA85218.

References

1. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, Riddell SR (1995) Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* 333:1038-1044.
2. Kalams SA, Walker BD (1998) The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J Exp Med* 188:2199-2204.
3. Knutson KL, Disis ML (2001) Expansion of HER-2/neu specific T cells ex vivo following immunization with a HER-2/neu peptide-based vaccine. *Clin Breast Cancer* 2:73-79.
4. Cohen PA, Kim H, Fowler DH, Gress RE, Jakobsen MK, Alexander RB, Mule JJ, Carter C, Rosenberg SA (1993) Use of interleukin-7, interleukin-2, and interferon-gamma to propagate CD4+ T cells in culture with maintained antigen specificity. *J Immunother* 14:242-252.
5. Webb LM, Foxwell BM, Feldmann M (1999) Putative role for interleukin-7 in the maintenance of the recirculating naive CD4+ T-cell pool. *Immunology* 98:400-405.
6. Cruikshank WW, Kornfeld H, Center DM (2000) Interleukin-16. *J Leukoc Biol* 67:757-766.
7. Trinchieri G (1994) Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 84:4008-4027.

8. Lee K, Overwijk WW, O'Toole M, Swiniarski H, Restifo NP, Dorner AJ, Wolf SF, Sturmhoefel K (2000) Dose-dependent and schedule-dependent effects of interleukin-12 on antigen-specific CD8 responses. *J Interferon Cytokine Res* 20:589-596.
9. McFarland EJ, Harding PA, MaWhinney S, Schooley RT, Kuritzkes DR (1998) In vitro effects of IL-12 on HIV-1-specific CTL lines from HIV-1- infected children. *J Immunol* 161:513-519.
10. Tuttle TM, Anderson BW, Thompson WE, Lee JE, Sahin A, Smith TL, Grabstein KH, Wharton JT, Ioannides CG, Murray JL (1998) Proliferative and cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients. *Clin Cancer Res* 4:2015-2024.
11. Disis ML, Grabstein KH, Sleath PR, Cheever MA (1999) Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin Cancer Res* 5:1289-1297.
12. Cheever MA, Chen W (1997) Therapy with cultured T cells: principles revisited. *Immunol Rev* 157:177-194.
13. Knutson KL, Schiffman K, Disis ML (2001) Immunization with a HER-2/neu Helper Peptide Vaccine Generates of HER-2/neu CD8 T Cell Immunity in Cancer Patients. *J Clin Invest* 107:477-484.
14. Rosenberg SA (2001) Progress in human tumour immunology and immunotherapy. *Nature* 411:380-384.

15. Hu HM, Winter H, Urban WJ, Fox BA (2000) Divergent roles for CD4⁺ T cells in the priming and effector/memory phases of adoptive immunotherapy. *J Immunol* 165:4246-4253.
16. Reilly RT, Gottlieb MB, Ercolini AM, Machiels JP, Kane CE, Okoye FI, Muller WJ, Dixon KH, Jaffee EM (2000) HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res* 60:3569-3576.
17. Surman DR, Dudley ME, Overwijk WW, Restifo NP (2000) Cutting edge: CD4⁺ T cell control of CD8⁺ T cell reactivity to a model tumor antigen. *J Immunol* 164:562-565.
18. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H (1998) The central role of CD4⁺ T cells in the antitumor immune response. *J Exp Med* 188:2357-2368.
19. Wilson CC, Olson WC, Tuting T, Rinaldo CR, Lotze MT, Storkus WJ (1999) HIV-1-specific CTL responses primed in vitro by blood-derived dendritic cells and Th1-biasing cytokines. *J Immunol* 162:3070-3078.
20. Marshall JD, Secrist H, DeKruyff RH, Wolf SF, Umetsu DT (1995) IL-12 inhibits the production of IL-4 and IL-10 in allergen-specific human CD4⁺ T lymphocytes. *J Immunol* 155:111-117.
21. Uherova P, Connick E, MaWhinney S, Schlichtemeier R, Schooley RT, Kuritzkes DR (1996) In vitro effect of interleukin-12 on antigen-specific lymphocyte proliferative responses from persons infected with human immunodeficiency virus type 1. *J Infect Dis* 174:483-489.

22. van Bergen CA, Smit WM, van Sluijters DA, Rijnbeek M, Willemze R, Falkenburg JH (1996) Interleukin-10, interleukin-12, and tumor necrosis factor-alpha differentially influence the proliferation of human CD8+ and CD4+ T-cell clones. *Ann Hematol* 72:245-252.
23. Gerosa F, Paganin C, Peritt D, Paiola F, Scupoli MT, Aste-Amezaga M, Frank I, Trinchieri G (1996) Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. *J Exp Med* 183:2559-2569.
24. Laster SM, Wood JG, Gooding LR (1988) Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. *J Immunol* 141:2629-2634.
25. Reece JC, Geysen HM, Rodda SJ (1993) Mapping the major human T helper epitopes of tetanus toxin. The emerging picture. *J Immunol* 151:6175-6184.

Footnotes

¹ Abbreviations: IVS, *in vitro* stimulation; PBST, phosphate-buffered saline containing Tween-20; ELISpot, enzyme-linked immunosorbent spot assay; HLA-A2, Human Leukocyte Antigen A2; ICD, intracellular domain.

Figure Legends

Figure 1. Generation of immunity to HER-2/neu helper peptide, p776-790, and HER-2/neu protein, ICD, in patients with HER-2/neu-overexpressing cancers. Patients were vaccinated with HER-2/neu peptides once a month for 6 months. Peptide-specific immunity was assessed prior to (Pre) and after vaccination (Post). T cell proliferation was assessed by ^3H -thymidine incorporation assays and stimulation index was calculated from 24-well replicates. The p776-790 (immunizing peptide) response is depicted by white bars, p42-56 (irrelevant, non-immunizing, HER-2/neu peptide) response by gray bars, and ICD response by black bars (protein). S.I.s greater than 2 (dotted line) are indicative of a positive proliferation response to antigens as described in the Materials and Methods.

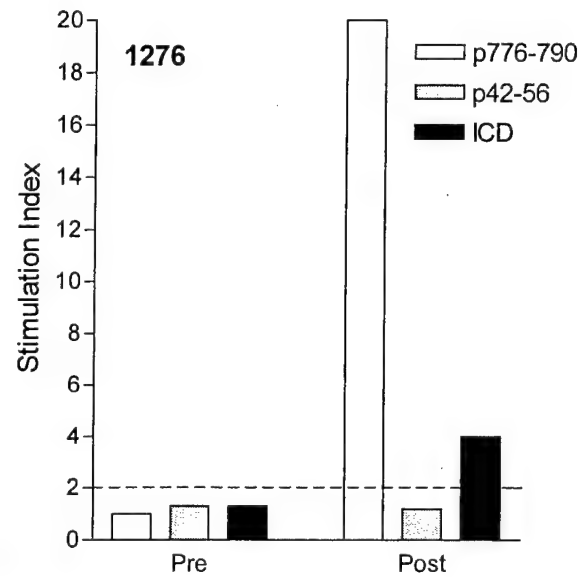
Figure 2: The addition of IL-12 to IL-2 increases antigen-specific clonal proliferation after IVS with HER-2/neu helper peptide. Panels A and B the proliferation responses to various concentrations of p776-790 (0-150 $\mu\text{g/ml}$) of cultures carried through 1 IVS in the presence of 10 $\mu\text{g/ml}$ p776-790, 10 U/ml IL-2 and varying concentrations of IL-12 (filled symbols). Panel A shows results with PBMC derived from patient 1276 and panel B from patient 6622. The concentration of IL-12 used during IVS is shown next to the corresponding lines. In panels C (patient 1276) and D (patient 6622), data is presented as the counts per minute (CPM) for proliferation responses to media alone (No antigen) or p776-790 following IVS with 10 $\mu\text{g/ml}$ p776-790, 10 U/ml IL-2, and varying concentrations of IL-12 from the same patients as indicated in graph.

Figure 3: The addition of IL-12 to IL-2 increases the CD4^+ T cell precursor frequencies after IVS with HER-2/neu helper peptide. PBMC from patient 1276 (panel A) and 6622 (panel B) were cultured with 10 $\mu\text{g/ml}$ p776-790 peptide in the presence of 10 U/ml IL-2 alone (open bars) or 10 U/ml

IL-2 and 10 ng/ml IL-12 (filled bars). Peptide-specific precursors were measured by IFN- γ ELISpot analysis following restimulation of the cultured cells with no antigen, p776-790, or the irrelevant HER-2/neu peptide, p42-56. Data is presented as the mean precursor frequency/million cells (\pm sem) calculated from 6 (panel A) or 3 replicates (panel B).

Figure 4: The addition of IL-12 to IL-2 increases peptide-specific TNF- α release by T cells after IVS with HER-2/neu helper peptide. PBMC from patient donors 1276 (panel A) and 6622 (panel B) were cultured with 10 μ g/ml p776-790, 10 U/ml IL-2, and varying concentrations of IL-12 (0, 1, and 10 ng/ml). The cells were subsequently tested for TNF- α release against no antigen (none, squares), 10 μ g/ml p776-790 (triangles) or 10 μ g/ml p42-56 (circles). Each determination is the average (\pm sem) for triplicate determination. * $p=0.004$; ** $p=0.04$, compared to TNF- α release from cultures carried through IVS with IL-2 alone and subsequently restimulated with 10 μ g/ml p776-790. The absence of an error indicates an standard error of the mean of less than 1%.

A



B

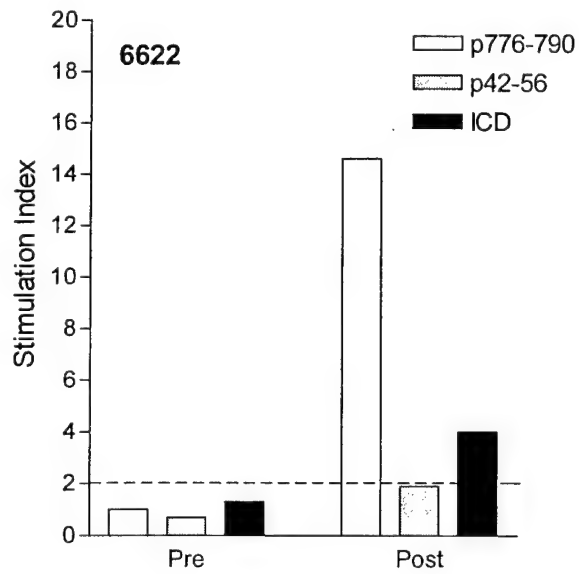


Figure 1

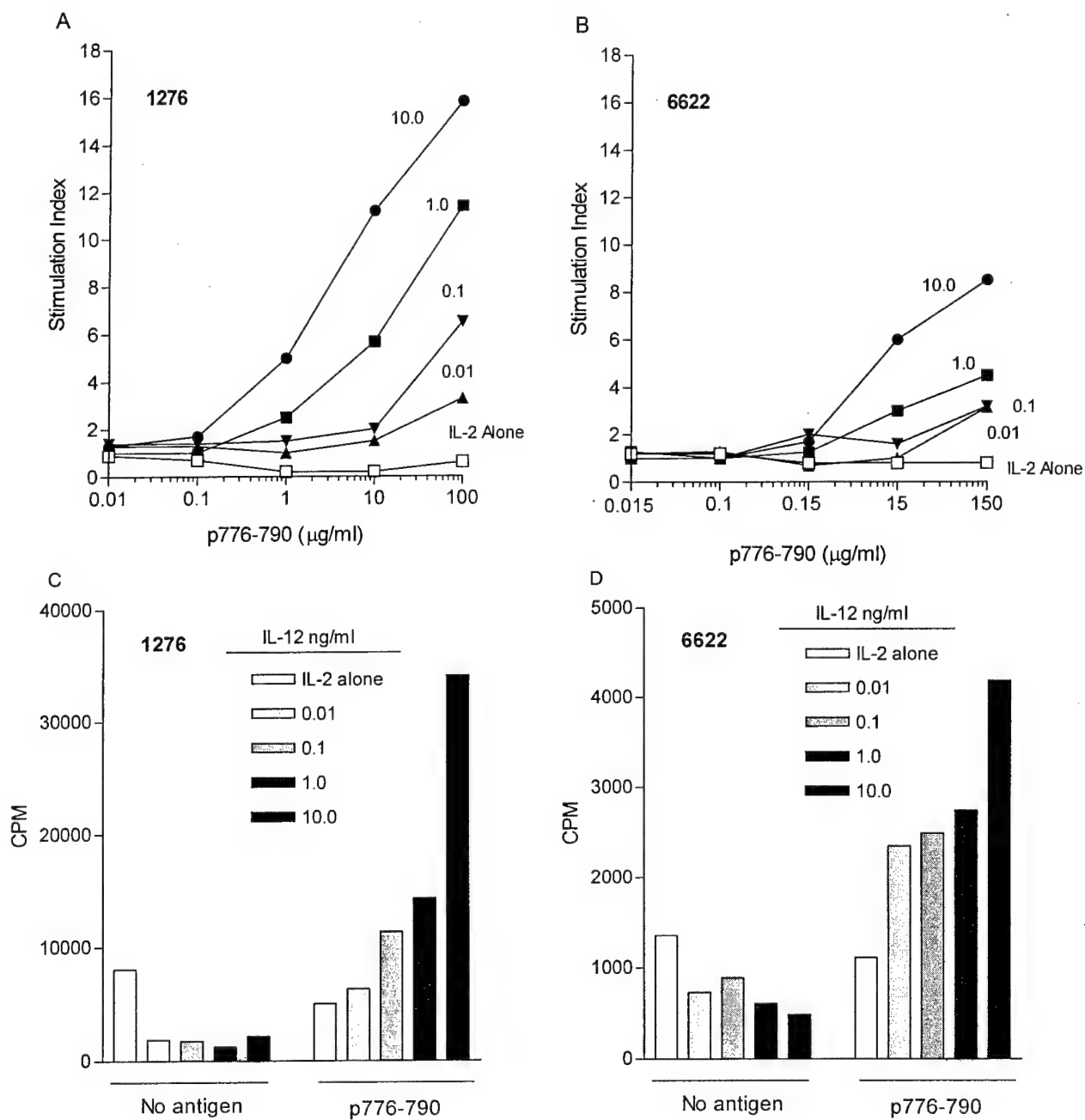


Figure 2

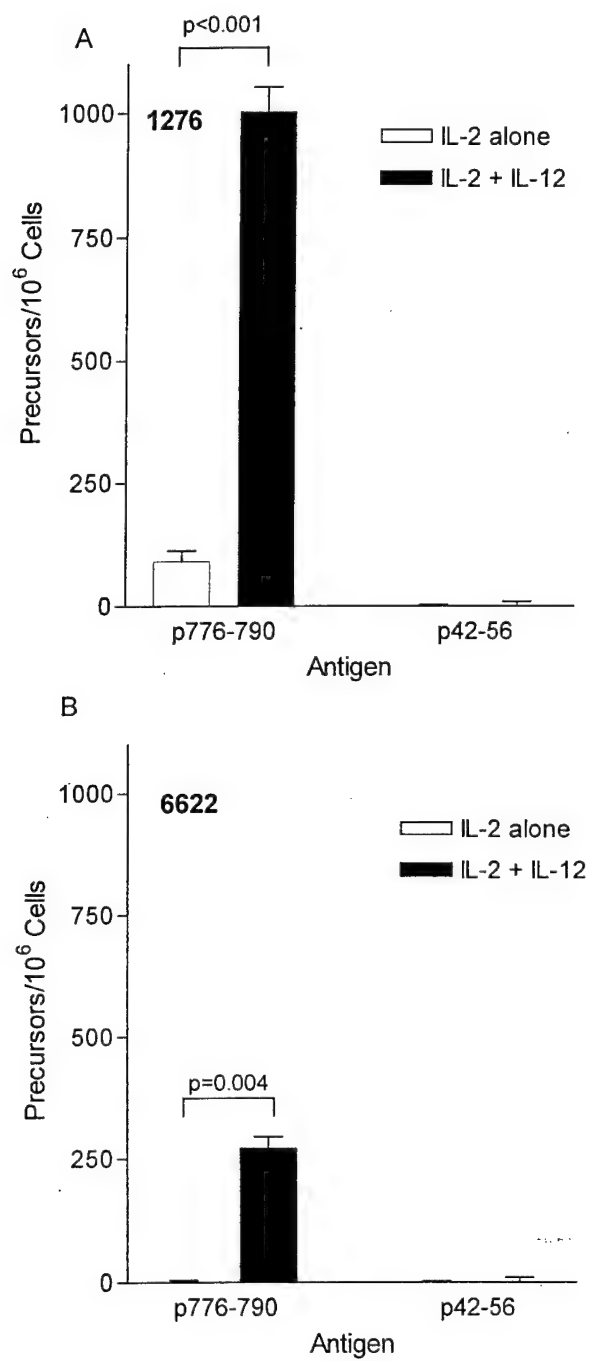


Figure 3

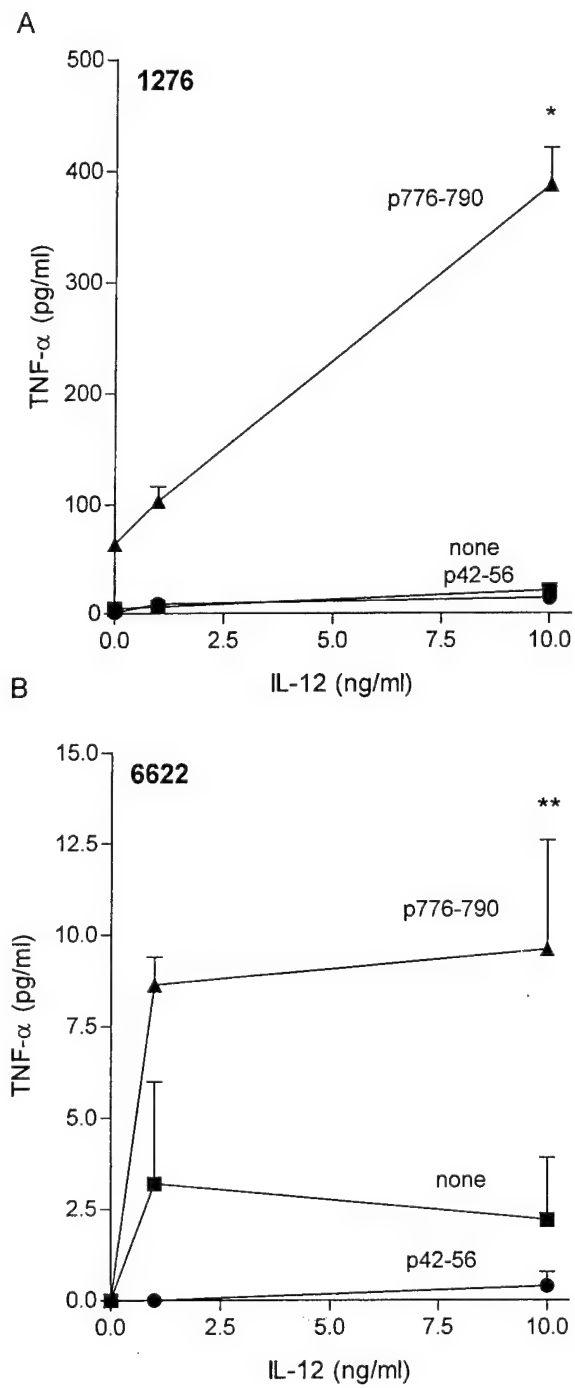


Figure 4

APPENDIX 4

Expert Opinion

1. Introduction
2. The clinical role of adoptive T-cell therapy
3. Human clinical trials of adoptive T-cell therapy
4. *Ex vivo* expansion of tumour-specific T-cells
5. Enhancing *in vivo* function and longevity of transferred T-cells
6. Applications of imaging to immunotherapy
7. Conclusion

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Adoptive T-cell therapy for the treatment of solid tumours

Keith L Knutson, Bond Almand, David A Mankoff, Kathy Schiffman & Mary L Disis

Division of Oncology and Division of Nuclear Medicine, University of Washington, Seattle, WA 98195, USA

Solid tumours can be eradicated by infusion of large amounts of tumour-specific T-cells in animal models. The successes seen in preclinical models, however, have not been adequately translated to human disease due, in part, to the inability to expand tumour antigen-specific T-cells *ex vivo*. Polyclonality and retention of antigen-specificity are two important properties of infused T-cells that are necessary for successful eradication of tumours. Investigators are beginning to evaluate the impact of attempting to reconstitute full T-cell immunity representing both major T-cell subsets, cytolytic T-cells and T-helper (Th) cells. One of the more important and often overlooked steps of successful adoptive T-cell therapy is the *ex vivo* expansion conditions, which can dramatically alter the phenotype of the T-cell. A number of cytokines and other soluble activation factors that have been characterised over the last decade are now available to supplement *in vitro* antigen presentation and IL-2. Newer molecular techniques have been developed and are aimed at genetically altering the characteristics of T-cells including their antigen-specificity and growth *in vivo*. In addition, advanced imaging techniques, such as positron emission tomography (PET), are being implemented in order to better define the *in vivo* function of *ex vivo* expanded tumour-specific T-cells.

Keywords: cytotoxic T-cells (CTL), cancer, cytokine, dendritic cells, *ex vivo* expansion, T-helper cell, polyclonal, T-cell receptor, vaccine

Expert Opin. Biol. Ther. (2002) 2(1):55-66

1. Introduction

Animal models demonstrate that adoptive T-cell therapy of advanced stage malignant disease is a feasible and successful treatment strategy. Increased understanding of the complex nature of the immune effector cells and the identification of tumour antigens is providing researchers with the appropriate tools to generate and reconstitute effective tumour-specific immunity through adoptive transfer of T-cells. *Ex vivo* expansion has been problematic and many hurdles will need to be overcome. Two advances in T-cell culture have improved the ability to generate tumour-specific T-cells *ex vivo*. The first was to increase antigen-primed T-cells *in vivo* prior to *ex vivo* expansion by active immunisation. The second was the improvement of culture conditions with the use of recently identified tumour antigens, cytokines and co-stimulatory molecules in conjunction with IL-2. In addition, technologies are being developed to genetically modify T-cells to create the appropriate immune microenvironment for tumour destruction. A better understanding of immunoregulatory mechanisms will allow us to overcome tumour-induced immunosuppression in cancer patients during adoptive T-cell therapy.

2. The clinical role of adoptive T-cell therapy

Adoptive T-cell therapy is an immune-based therapeutic strategy that will significantly boost tumour-specific T-cell immunity above that observed by vaccination alone [1]. Transferred T-cells can potentially represent a major fraction (1:2) of the host's lymphocytes [1]. This strategy could have an advantage over active vaccination for the treatment of more extensive malignancy where greater control of the numbers of tumour-specific T-cells may be required. Adoptive T-cell therapy techniques that have shown great promise in human clinical trials have been applied to the Epstein-Barr virus (EBV)-related disorders, immunoblastic lymphoma and Hodgkin's disease [2-4]. These studies, as will be further discussed, provide proof of principle that administration of *ex vivo* expanded T-cells can reconstitute effective long lasting immunity *in vivo*. However, the major distinction between the treatment of EBV-malignancies and non-viral malignancies is the targeting of non-self viral antigens. In the most common solid tumours, self proteins are the target antigens of T-cells. Thus, *ex vivo* expansion of these T-cells is further complicated by the probability that the most potent and robust self tumour antigen-specific T-cells recognising self antigens have either been deleted or rendered ineffective by tolerising mechanisms [5,6]. *Ex vivo* expansion methods must be optimised to maximise functional effects of the remaining self-reactive T-cells while preventing the outgrowth of non-specific immune effector cells. Recent evidence demonstrating that single clones are ineffective at mediating tumour eradication further suggests that *ex vivo* expansion methods should also be designed to maintain polyclonality in order to ensure multiple specificities for the same epitope as well as multiple T-cells subsets specific for the target antigen [7]. The need for polyclonal responses is particularly important for a number of reasons, including the prevention of antigen-loss variants, the prevention of major histocompatibility complex (MHC) class I loss variants and the need of helper activity by cytotoxic T-cells (CTL) for expansion and persistence *in vivo*. Much of the current thinking of the obstacles of adoptive T-cell therapy stems from mouse models and the results of previously reported human clinical trials.

3. Human clinical trials of adoptive T-cell therapy

Clinical trials of adoptive T-cell therapy arose from promising earlier studies evaluating the antitumour efficacy of lymphokine activated killers (LAK). LAK are generated from patient peripheral blood mononuclear cells (PBMC) with IL-2 and are capable of killing tumour cells in a non-MHC-restricted fashion. Clinical trials, utilising LAK, have been carried out by several groups treating a variety of carcinomas, including melanoma, ovarian, renal cell and colorectal [8-10]. The low response rates ranging from 0 - 20% following infu-

sion of LAK are likely a reflection of the inability of the LAK to home to tumour sites and the lack of specificity for the tumour. However, the response rates were encouraging and provided impetus for identifying lymphocyte populations, mainly T-cells, that have increased specificity and antitumour effector function.

The strategies which have been developed for increasing the number of tumour-specific T-cells, *ex vivo*, have included the non-specific expansion of tumour infiltrating lymphocytes (TIL), the non-specific expansion of sensitised lymph nodes draining the tumour site and the expansion of tumour antigen-specific T-cell populations. The first two strategies are based on the hypothesis that there is an increased precursor frequency of tumour-specific T-cells at sites local to the tumour or in the lymph node draining the tumour. The last strategy exploits the use of known tumour antigens to expand specific T-cell populations.

Beginning in the late 1980s, clinical trials were carried out using *ex vivo* expanded TIL from a variety of cancers including malignant melanoma, renal cell carcinoma, breast cancer and lung cancer. Response rates after TIL therapy were variable and ranged between 0 - 60% with most being between 10 - 25% [11]. Studies using TIL have been difficult to perform in cancer patients due to:

- The limitations in obtaining significant amounts of tumour from which to derive the cells.
- The inability to expand autologous cancer target cell lines to test T-cell lytic activity prior to infusion.
- The location of metastatic relapse (e.g., bone, lung, brain and liver) prevents tumour cell and thus T-cell harvest.

TIL are typically expanded by incubating the cells with very high concentrations, up to 7000 U/ml, of IL-2. These culture conditions typically result in enriching for T-lymphocytes with LAK-like activity. For example, Beldegrun and colleagues reported the results of a study characterising the cell populations derived from *ex vivo* expansion of lymphocytes infiltrating human renal cell cancer [12]. Renal TIL expanded in high doses of IL-2 are predominantly CD3+ but display lytic activity similar to LAK, including high activity against K562, Daudi and allogeneic tumour cells. Ratto and colleagues observed similar findings following expansion of lung TIL [13].

The use of IL-2-expanded TIL in melanoma trials has led to observations that could have important implications for the design of future adoptive T-cell trials. Rosenberg and colleagues reported the results of a clinical trial evaluating treatment of melanoma patients with both TIL and high dose bolus IL-2 [14,15]. Of the 86 patients treated, 24 partial and five complete responses were observed. A number of important correlations were observed when comparing the *in vitro* characteristics of the expanded TIL of responders with the TIL of the non-responders. Clinical responses were associated *in vitro* cellular responses such as higher specific lysis of autologous tumour targets, shorter doubling times, younger cultures and

increased autologous tumour-specific granulocyte-macrophage colony stimulating factor (GM-CSF) secretion [15].

Like TIL, lymph nodes draining either a tumour or vaccine site represent another potentially rich source of tumour-specific T-cells. Clinical response rates following reinfusion of *ex vivo* activated nodal T-cells are similar to TIL infusions [11]. In a recent Phase I study, To and colleagues evaluated the toxicity and clinical responses of infusions of *ex vivo* expanded vaccine-draining lymph node-derived lymphocytes in patients with head and neck cancers [16]. Fifteen patients were vaccinated on the thigh with irradiated autologous tumour with GM-CSF. After 8 - 10 days, the inguinal, vaccine-draining lymph nodes were harvested and activated *ex vivo* with staphylococcal enterotoxin A (SEA) and expanded in high dose IL-2. The resulting cells were mainly CD3+ and had mixed CD8+ and CD4+ phenotype. Toxicity following reinfusion was minimal and limited to grade 2. Of the 15 patients, only two responded with one patient being disease free. In a similar study, patients with newly diagnosed gliomas were immunised with autologous tumour cells followed by harvesting and expansion of the vaccine-draining lymph nodes [17]. The trial resulted in four partial responses out of 12 patients treated. Further *in vitro* characterisation of the resulting T-cell populations can provide more insight into how this promising technique can be optimised. As an example, perhaps increased tumour killing could be achieved by including specific tumour antigens during expansion.

Recent advances in molecular and cellular immunology provide the technology needed to identify and define tumour-specific antigens, as well as an understanding of how T-cells recognise antigens. It is presumed that a highly purified, antigen-specific and polyclonal T-cell population would be the most efficient strategy for tumour eradication. Clinical trials testing the feasibility of antigen-specific T-cell therapy for the treatment of disorders and cancers associated with EBV have been reported. EBV-specific CTL lines have been generated *in vitro* from donor blood and used successfully to treat B-cell lymphoproliferative disorder in bone marrow transplant recipients [4]. In addition, EBV-specific CTL have been expanded *ex vivo* and reinfused into patients with advanced stage relapsed Hodgkin's disease [2]. This feasibility study demonstrated:

- CTL could be expanded from patients with advanced cancer.
- T-cells were found to persist up to 13 weeks after infusion.
- Antigen-specific T-cells were detected in the pleura at levels 10-fold higher than the peripheral blood, implying trafficking of infused T-cells to sites of tumours.
- Transferred T-cells produced increased EBV-specific cytotoxic activity in peripheral blood lymphocytes as measured by chromium release assay and a decrease in peripheral blood viral burden as measured by quantitative PCR.

A preliminary report of the clinical status of patients receiving this treatment indicated that in addition to reducing viral burden, the T-cell infusion may stabilise or reduce disease symp-

toms [18] suggesting that Phase II clinical trials should be carried out to evaluate clinical responses.

Recently, Dudley and colleagues assessed the safety, feasibility and clinical response of adoptive T-cell therapy of melanoma using *ex vivo* expanded CD8+ cytolytic T-cell clones specific for a human lymphocyte antigen (HLA)-A2 binding peptide derived from the melanoma antigen GP-100 [7]. Twelve patients were treated with multiple infusions of GP-100-specific T-cell clones with an average of 1×10^{10} cells/infusion. The T-cells clones were selected based on their apparent avidity for peptide antigen as assessed by the magnitude of antigen-specific cytokine release. Although the clones secreted large amounts of IFN- γ and recognised HLA-A2+ melanoma cell lines, only two patients had minor partial responses. An important finding from this study was that the T-cells disappeared rapidly and were undetectable at 2 weeks, even though the patients received concomitant iv. IL-2. The authors postulated several mechanisms to explain the lack of clinical effects. One possibility is that the CD8+ T-cell clones could not persist in the absence of sufficient help, and the authors suggested that the treatment could be improved by co-infusion with antigen-specific CD4+ T-cells. This is supported by previous studies by Walter and colleagues who observed that cytomegalovirus (CMV)-specific CTL clones declined more rapidly in patients deficient in CD4+ Th cells specific for CMV [19].

These clinical trials as well as many others identify major areas that should be further investigated to improve the clinical outcome of adoptive immunotherapy. Namely, identification of *ex vivo* expansion conditions and other novel molecular techniques aimed at improving the antigen-specificity, functionality, polyclonality and longevity of the infused T-cells.

4. *Ex vivo* expansion of tumour-specific T-cells

The success in expanding EBV-specific T-cells from peripheral blood of patients may be related to the endogenous, naturally occurring high precursor frequency of these cells, thus, the abundance of viral-specific T-cells in the initial cultures. In contrast to viral cancers, T-cells directed against non-viral tumours are much less abundant [20]. *Ex vivo* expansion of tumour antigen-specific T-cells may be greatly facilitated by prior immunisation against specific tumour antigens. The authors recently demonstrated the feasibility of this approach in breast and ovarian cancer patients who were vaccinated against HER-2/neu with a helper peptide-based vaccine [21]. Nineteen patients were vaccinated with a HER-2/neu peptide-based vaccine consisting of three helper peptides, each of which contained an HLA-A2 binding motif, fully nested within its sequence. Six monthly vaccinations with GM-CSF as adjuvant resulted in increased levels of T-cells specific for the encompassed HLA-A2 motifs to levels similar to those measured against influenza and CMV HLA-A2-binding peptides. The increased precursor frequency after vaccination improved the generation of T-cell clones specific for the

HER-2/neu HLA-A2 binding peptide, p369-377. As an example, in one patient, a total of 21 p369-377 clones were generated. With the exception of two clones, all clones were CD3+ [22]. Eleven of the clones were CD8+/CD4-. Nine of the clones were CD4+/CD8-, despite being specific for an HLA-A2 binding peptide. The remaining five clones contained varying levels of both CD4+ and CD8+. The majority (19/21) of clones expressed the α/β T-cell receptor but interestingly, two clones expressed the γ/δ T-cell receptor [23]. Several of these clones could be induced to secrete IFN- γ in response to p369-377 peptide stimulation. Several clones were able to lyse HLA-A2-transfected HER-2/neu-overexpressing tumour cells, including the γ/δ T-cell receptor (TCR) expressing clones. Similarly, Reddish and colleagues demonstrated that breast cancer patients can generate MHC class I-restricted CTL against MUC-1-expressing adenocarcinomas following vaccination with a MUC-1 helper peptide [24]. Investigations such as these demonstrate that *ex vivo* expansion and characterisation of cancer specific T-cells is facilitated by vaccination and that the responses elicited to vaccine can be diverse and polyclonal.

Several techniques for *ex vivo* expansion of tumour-antigen T-cells are currently being developed in various laboratories. Two variables that can be manipulated during *ex vivo* expansion are the antigen and cytokine environments. The identification of tumour-specific antigens, as well as the important tumour-responding T-cell populations, has ushered in a new era of cellular expansion techniques that can generate T-cell lines and clones with increased antigen-specificity. Manipulating the cytokine environment also allows for the preferential expansion of T-cell subsets such as CD4+ and CD8+ or Th1 and Th2. Antigen-specific techniques are preferable over non-antigen-specific techniques due to the fact that even with vaccination, antigen-specific T-cell precursors may not be at sufficient levels to expand preferentially during stimulation with non-specific activation such as with anti-CD3/anti-CD28 beads or bacterial products such as SEA. This is evident from previous studies demonstrating that expansion of tumour-infiltrating T-cells with non-specific methods does not promote expansion of tumour-specific T-cells that are therapeutic *in vivo* [16,17]. These methods may activate all T-cells to a similar degree and may result in the expansion of non-specific bystander T-cells, immunosuppressive T-cells, or tolerated, non-functional T-cells. Methods are now being established allowing for selective expansion of specific T-cell subsets.

Adoptive T-cell therapy strategies have largely focused on the *ex vivo* generation of CTL due to observations that most tumours express MHC class I but not MHC class II and that CTL can mediate tumour regression in mice. Studies demonstrating the weak persistence of transferred CTL have led to investigations on how to extend their lifespans. Recent evidence from our laboratory suggests that simultaneous generation of tumour antigen-specific CD4+ Th cells could prolong the life of CTL *in vivo* [21]. The authors observed that > 60% of patients immunised with HER-2/neu helper epitopes, each

containing an encompassed HLA-A2 epitope, were able to develop HER-2/neu specific CD8+ T-cell immunity. The CD8+ T-cell response was maintained, in some patients, for at least one year following vaccination. In contrast, 2/5 (40%) patients immunised with a single HER-2/neu HLA-A2 9-mer peptide, p369-377 (E75) developed HER-2/neu CD8 T-cell immunity that declined to undetectable levels within 5 months of the last vaccination (Knutson & Disis, unpublished observations). These data are consistent with findings in murine viral models where persistence of CD8+ T-cells is critically dependent on concurrent CD4+ T-cell immunity [25]. For example, in the murine LCMV model of viral immunity, loss of CD4 T-cell help results in impaired memory phase CTL responses leading to the inability of the mice to permanently control infection [26-28]. Moreover, CD4+ T-cells may also possess direct and indirect killing properties [29]. CD4+ T-cells encountering tumour directly or indirectly through dendritic cell (DC) cross-presentation can release a wide variety of cytokines, such as TNF-related apoptosis-inducing ligand (TRAIL), which can activate apoptotic pathways in tumour cells [30,31].

The *ex vivo* expansion of tumour antigen-specific CD4+ T-cells has been impeded by the lack of defined MHC class II-restricted tumour antigen peptides and the appropriate cytokine environment optimal for the generation of CD4+ T-cells capable of eliciting an inflammatory or Th1 type response. The authors had previously identified several MHC class II peptides, derived from HER-2/neu [32]. It was determined which of these peptides were relevant immunogens based on their ability to induce T-cells that recognised naturally processed HER-2/neu protein antigens in breast, ovarian and lung cancer patients [33]. Thus, with the identification of relevant MHC class II antigens, an important objective was to define the appropriate cytokine environment that preferentially promotes the expansion of HER-2/neu-specific CD4+ T-cells with a Th1 type profile, a phenotype important to the development of a cell-mediated immune response at the tumour site. Th1 cells could be efficiently cultured by the inclusion of IL-12 along with peptide and IL-2 during culture. IL-12 is a heterodimeric cytokine, produced by B-cells, macrophages and professional antigen presenting cells (APC), that has multiple effects on CD8 T-cell function when added together with low-dose IL-2 [34-39]. As a model MHC class II antigen, the authors chose p776-790 derived from the intracellular domain of HER-2/neu. The majority of patients immunised with this peptide developed immunity to HER-2/neu protein. Furthermore, this epitope was commonly associated with epitope spreading suggesting natural presentation [40]. While immunity to p776-790 could be readily measured in short-term cultures, cell lines generated by *in vitro* stimulation with peptide and IL-2 as the only added cytokine resulted in no antigen-specific expansion. The inclusion of IL-12, along with IL-2, restored antigen-specific responsiveness in a dose-dependent fashion [41]. The resulting p776-790-specific T-cells responded readily to antigen by proliferat-

ing and producing Type I cytokines (IFN- γ and TNF- α). The increased proliferative response of the cultures was due in part to an increase in the number of HER-2/neu-specific T-cells as assessed directly by enzyme-linked immunoSPOT (ELISPOT) analysis. Inclusion of IL-12 into the cultures also resulted in a significant decrease of non-specific cellular proliferation. These results suggest that IL-12 is an important cytokine for *ex vivo* recovery and maintenance of antigen-specific CD4⁺ T-lymphocytes that would otherwise be lost by using IL-2 as the only cytokine.

IL-7 has shown promise for the expansion of CTL under certain *ex vivo* conditions. IL-7 is a stromal cell-derived cytokine and is associated with the early development of lymphoid cells. IL-7 activates the proliferation of naive T-cells and has been implicated as a key cytokine in maintaining homeostatic proliferation *in vivo* [42]. Recent findings suggest that the addition of IL-7 to cultures can promote preferential expansion of antigen-specific T-cells [43]. TIL derived from follicular lymphoma (FL) typically lack tumour-specific activity which is not recovered by culturing cells with FL along with IL-2. However, when TIL are preactivated through CD40 followed by exposure to FL they can be further expanded by inclusion of IL-7 along with IL-2. The expanded T-cells have greatly enhanced FL-specific CTL activity. The effects of IL-7 appear however to depend on the *ex vivo* expansion environment. In the lab, the authors have found that when IL-7 is included along with an influenza matrix peptide and IL-2, peptide-specific lysis is reduced by 25–30% compared to cells cultured with peptide and IL-2 alone (Knutson, unpublished observations). The background, non-specific lysis was also increased 3-fold. This lack of effect of induction of peptide-specific responses may not be translatable to other peptide systems. For example, Tsai and colleagues have found that IL-7 potentiated the ability of peptide-pulsed DCs to generate CTL responses against viral and tumour epitopes [44]. These discrepancies in outcome clearly point to the need to optimise the use of cytokines in preclinical studies prior to clinical trials.

Recent studies with IL-15 demonstrate that this cytokine can have important effects on *ex vivo* expansion of peptide- and protein-specific T-cells. IL-15 is structurally similar to IL-2 and their receptors share the IL-2R β and IL-2 γ chains [45]. The IL-2R α (CD25) and IL-15R α chains confer specificity. Like IL-2, IL-15 is a pleiotropic cytokine and induces proliferation and functional changes of multiple haematopoietic cells including $\alpha\beta$ T-cells, $\gamma\delta$ T-cells, DC and natural killer cells [46]. IL-2 predisposes T-cells to undergo activation-induced cell death (AICD) and IL-15 promotes the generation of memory CD8⁺ T-cells [47]. The death-inducing effects of IL-2 are particularly important for the expansion of CD4⁺ T-cells which are extremely sensitive to IL-2 following antigen stimulation. IL-2 and IL-15 also can change the homing capabilities of cultured CD8⁺ T-cells [48]. Antigen-primed murine CD8⁺ T-cells cultured in IL-15 but not IL-2, preferentially home to lymphoid tissue such as spleen and lymph

nodes, while IL-2 cultured CD8⁺ T-cells home to sites of inflammation but not lymphoid tissue. IL-15 cultured cells home to sites of inflammation to a lesser extent but mediate a robust antigen recall response. Currently it is unknown if these findings can be extrapolated to human T-cells but could have important implications in designing expansion conditions to generate T-cells capable of targeting lymph node disease. Pharmacological generation of an inflammatory response with anti-apoptotic agents may be considered in order to attract IL-2-cultured cells to tumour site.

Activation of T-cells directly through CD40 or indirectly using trimeric CD40L also enhances the expansion of antigen-specific T-cells [49]. CD40L is expressed on T-cells and is the ligand for CD40 which is expressed on antigen-presenting cells, including DCs [50]. The interaction of CD40 with CD40L is IL-2- and CD28- independent and results in increased proliferation of T-cells as well as increased expression of Th1 cytokines (IFN- γ , TNF- α) [49]. During induction of tumour-specific immunity, CD40 activation promotes long-term survival of tumour-specific CD8⁺ T-cells [51]. Terheyden and colleagues have recently reported encouraging results demonstrating the utility of CD40 ligation during *ex vivo* expansion of melanoma-specific CD8⁺ T-cells and CD4⁺ T-cells derived from TIL [52]. When T-cells were cloned from TIL using oncolysate-pulsed DCs, the resulting phenotype of the cell population was predominantly CD4⁺ Th2-like cells demonstrating high IL-4 production. The inclusion of anti-CD40 monoclonal antibody (mAb) to ligate CD40 on autologous DCs induced both CD8⁺ and CD4⁺ T-cells that were specific for melanoma antigens. The CTL had high lytic activity against autologous tumour but not allogeneic tumour nor autologous fibroblasts. While many of the CD4⁺ T-cell clones were exclusively Th1 (IFN- γ +, IL-4-), many possessed a Th0 (IFN- γ +, IL-4+) phenotype. The clinical significance of these latter Th0 cells are currently unknown but newer findings in murine models of adoptive T-cells therapy suggest that better tumour eradication is achieved with both cell-mediated and humoral immunity [53].

DC-based T-cell expansion methods are an attractive approach because DCs produce many of the necessary soluble activation factors for *ex vivo* expansion of antigen-specific T-cells. Several DC-based strategies are currently being developed for expansion including loading DCs with apoptotic tumour cells, tumour antigen peptides, protein, or tumour cell-derived RNA. DCs are the most potent antigen-presenting cells of the immune system and are responsible for initiating and modulating the immune response [54]. Unlike tumour cells, DCs express co-stimulatory molecules and MHC class I and class II at high levels, and only a small number of DCs are required to activate and expand tumour-specific T-cells.

The frequency of restimulation as well as the source of antigen appear to be important for determining the phenotype of the resulting T-cell population when using DC-based expansion. While repeated weekly stimulation of PBMC with renal cell carcinoma-loaded DCs resulted in a predominant expan-

sion of CD4⁺ T-cells, alternating between tumour cell loaded DCs and irradiated tumour cells alone resulted in enriched CD8⁺ T-cells with potent specificity for renal cell lines [55]. Preferential expansion of tumour-specific CD8⁺ T-cells can be accomplished by prior enrichment with reagents such as peptide MHC class I tetramers [56] before stimulation with tumour cell- or antigen-pulsed DCs. DCs can also be transfected with tumour-derived RNA as has recently been reported by Heiser and colleagues for the *ex vivo* generation of polyclonal T-cells against prostate and renal cell carcinoma [57,58].

Tumour cell or tumour RNA isolation can be avoided by transfecting or transducing DCs with whole tumour antigen DNA, an approach that is attractive because it obviates the need to tailor make peptide antigens specific for the patient's MHC haplotype. This approach has been used by Bushenfelde and colleagues to generate CTL and Th cells specific for HER-2/neu using a retroviral transduction of DCs [59]. DCs were generated from CD34⁺ stem cells derived from patients and were transduced to express the full length HER-2/neu. Patient PBMC were then stimulated with the HER-2/neu-expressing DCs. After 3-weekly stimulations, both CTL and Th cells could be individually cloned. The clones recognised different regions of the HER-2/neu suggesting the capacity of the DCs to express multiple antigenic peptides. An important attribute of this approach is that the transduced DCs do not express high levels of HER-2/neu but rather moderate to low levels, which would support the generation of high affinity T-cells which would in turn, be able to target tumour cells with a broad range of antigen expression [59].

While *ex vivo* expansion techniques can be optimised to produce T-cell lines with improved antigen-specificity and function, as assessed by *in vitro* assays, the ultimate goal is to carry those attributes into the host following transfer. The complex *in vivo* environment can present new challenges to the T-cells that may not have been present *in vitro*. For example, the immune microenvironment contains immunosuppressive factors which will inhibit the growth and function of transferred cells. Methods are being developed to improve the function of T-cells *in vivo* and to overcome these obstacles.

5. Enhancing *in vivo* function and longevity of transferred T-cells

In vivo supplementation with soluble T-cell growth factors can promote the activation and longevity of adoptive transferred T-cells. For example, murine models of adoptive T-cell transfer have demonstrated that administration of IL-2 following adoptive transfer maintains high levels of precursor specific for viral antigens for extended periods of time [1]. This is consistent with studies in IL-2 knockout mice where frequencies of transferred ovalbumin-specific CD8⁺ T-cells declined significantly with time compared to the same T-cells injected into normal mice [60]. However, the toxicity of IL-2 in cancer patients limits its use and investigations are underway to identify less toxic strategies for the chronic maintenance

of transferred T-cells. Recent findings suggest that *iv*. IL-2 alone may not be sufficient to extend the life of transferred melanoma-specific CD8⁺ T-cell clones, suggesting the need for additional factors [7]. Recent findings by the authors demonstrate that HER-2/neu-specific CD8⁺ T-cells can persist for at least a year when generated concurrently with HER-2/neu-specific CD4⁺ T-cells suggesting that co-infusion of antigen-specific CD4⁺ T-cells along with CD8⁺ T-cells during adoptive T-cell therapy may improve CD8⁺ T-cell longevity [21]. Alternatively, it would be beneficial to identify other pharmacological means of improving function and extending T-cell life *in vivo*.

One candidate receptor for *in vivo* modulation of infused T-cells is the OX-40 receptor (OX-40R). OX-40R is a transmembrane receptor expressed predominantly on activated CD4⁺ T-cells and is a member of the TNF receptor superfamily. The ligand for OX-40, OX-40L, is expressed on activated APC and B-cells. *In vivo* engagement of OX-40R with OX-40L during tumour priming results in enhanced tumour immunity through increased activation of the endogenous antitumour CD4⁺ T-cell response [61,62]. These responses were observed for a variety of murine tumours. Recently, OX-40R activation using OX-40 mAb has been applied to adoptive T-cell therapy of 10-day MC205 pulmonary metastases and intracranial tumours. In that study it was found that administration of OX-40R mAb resulted in the need for significantly fewer tumour-specific T-cells to cure established malignancy [63]. *In vivo* T-cell trafficking studies revealed that the OX-40R mAb application did not result in an increased number of T-cells trafficking to tumour sites suggesting that OX-40R stimulation results in enhanced function of tumour-specific T-cells.

Methods of genetic modification of T-cells to enhance cellular function, deliver therapeutic factors, or enhance T-cell longevity will likely play a key role in the success of adoptive T-cell therapy in tumour eradication [64]. The feasibility of these approaches has been demonstrated in the murine models, experimental autoimmune encephalitis (EAE) and non-obese mouse diabetes (NOD). It is clear that inflammatory Th1 CD4⁺ T-cells are pivotal in the development of both EAE and NOD [65,66]. Encephalitogenic or diabetogenic Th1 T-cells can be genetically modified to deliver immunosuppressive cytokines which can limit the extent of the disease [67]. For example, delivery of IL-4 by retrovirus-transduced encephalitogenic T-cells delays onset and reduces severity of EAE induced by immunisation against myelin basic protein [68]. Similarly, islet-specific Th1 lymphocytes, transfected to express IL-10, prevent adoptively-transferred diabetes in NOD mice [69]. These results demonstrate that tissue-specific T-cells can be altered genetically to skew the Th1/Th2 environment, ultimately changing the course of the autoimmune disease. The genetic alterations of T-cells specific for cancer would take reverse strategy. Tumour-specific T-cells could be manipulated to increase the Th1 type response at the site of tumour to fur-

ther enhance inflammation. Target cytokines for overexpression might include IFN- γ , TNF- α and IL-2.

Tumour-specific T-cells can also be genetically engineered to control their *in vivo* growth capabilities. Since many human tumours grow slowly, treatment will likely require a sustained T-cell response to ensure eradication of all micrometastases as well as destruction of the primary tumour. Transferred T-cells, particularly CTL, are extremely short-lived in the absence of supplemental help and stimulation as previously discussed [7]. Aimed at improving the ability to manipulate the *in vivo* growth of tumour-specific T-cells, Evans and colleagues have developed a chimeric GM-CSF/IL-2 receptor which, when transduced into CTL, results in GM-CSF-sensitive proliferation mediated through the IL-2 signalling mechanism [70]. It is envisioned that systemic delivery of GM-CSF could be used to expand only transduced T-cells while avoiding the toxicities associated with IL-2 administration. In addition, the receptor is constitutively expressed and subject to local autocrine activation within the immune microenvironment.

Redirecting T-cell antigen-specificity is also possible using several different methods. One method is to transfect a TCR with known specificity into naive T-cells. As an example, Calogero and colleagues transduced Jurkat T-cells to express an HLA-A2-restricted $\alpha\beta$ -TCR gene specific for a MAGE 3 peptide [71]. The resulting T-cell line was activated in response to both T2 cells and a melanoma cell line loaded with MAGE 3 peptide. A limitation of this technique is that in many cancers, TCRs and antigens are not adequately defined. However, in cases where antigens and TCRs are known, this technique, potentially, could be applied to naive non-specific T-cells following inactivation of their endogenous TCR genes.

Another retargeting method is to produce signalling receptors containing an extracellular antigen-specific antibody fused to an intracellular domain that is able to mediate T-cell activation. In one recent study, chimeric receptors containing an extracellular antibody and the Fc receptor γ signalling chain have been made against the colon cancer-associated protein epithelial glycoprotein (EGP) 40 [72]. This chimeric receptor, GA733, when transduced into human T-cells conferred both cytokine production and cytolytic activity against EGP40+ colon cancer cells. In another study, Rossig and colleagues recently reported the generation of a T-cell line with dual recognition for EBV antigens and the neuroblastoma ganglioside antigen, GD2 [73]. EBV-specific T-cell lines were transfected with a construct containing the TCR- ζ -chain fused to variable domains of an anti-GD2 antibody. The resulting cells could potentially be maintained *in vivo* and *in vitro* with autologous EBV-infected cells.

In addition to enhancing T-cell function, immunosuppression may need to be circumvented during adoptive T-cell therapy. Infused tumour-specific T-cells, like endogenous tumour-specific T-cells, are likely targets of active systemic immunosuppression. Although multiple mechanisms of immunosuppression have been identified, recent investigations have focused extensively on T-regulatory cells (Tregs) [74-78], which

play a key role in the maintenance of immune tolerance to self antigens. Tregs constitute a homogenous population of CD4+ CD25+ T-cells and are selected for in the thymus by self peptides *via* high affinity TCRs [79]. Tregs represent up to 6% of circulating PBMC in humans [75-77]. Selection of Tregs represents an alternative to clonal deletion of T-cells with high affinity TCRs against self antigens. Tregs directly inhibit the growth and function of antigen-specific T-cells by direct cell-to-cell contact [75-77], and recent findings suggest that Tregs proliferate in response to IL-2 in the absence of TCR stimulation. In addition to a direct suppressive effect on T-cells, Tregs have also been shown to down-modulate the co-stimulatory molecules, CD80 and CD86, on APC [80].

Studies in murine cancer models have suggested a role for Tregs in mediating evasion of tumour cells from immune destruction. Onizuka and colleagues demonstrated that rejection of tumour could be enhanced against 6/8 different murine tumours, including leukaemia, myeloma and sarcomas by depleting Tregs [81]. In that study, tumours were rejected in mice that had been previously treated with a single bolus dose of anti-CD25 antibody 4 days earlier. The injections of anti-CD25 antibody resulted in significantly reduced levels of circulating CD25+ T-cells. Similar findings were reported by Shimizu and colleagues who demonstrated that depletion of CD25+ T-cells results in the endogenous generation of antitumour immunity that is directed against a broad spectrum of tumours [82]. These encouraging findings that Tregs may play a key role in suppressing endogenous murine antitumour immunity have led investigators to examine the role of Tregs in mediating evasion of human tumours. Woo and colleagues recently reported evidence for increased levels of Tregs associated with both non-small cell lung cancer (NSCLC) and late-stage ovarian cancer [78]. Compared with autologous peripheral blood T-cells, there was an increased number (approximately 1.5 - 2.0-fold) of CD25+ CD4+ T-cells associated with either the tumour-infiltrating lymphocytes of NSCLC or tumour-associated lymphocytes of ovarian cancer. The CD25+ CD4+ T-cells were isolated and examined for cytokine release and found to secrete an immunosuppressive cytokine, TGF- β . Overcoming the immunosuppressive properties of these cells by direct inhibition or by swamping the effects of Tregs by a variety of mechanisms could be an important objective in designing therapeutically effective adoptive T-cell transfer. In the same study, it was also observed that the ovarian cancer patients, but not the NSCLC patients, had increased levels of CD25+ CD4+ T-cells within the peripheral blood compartment suggesting that strategies may need to be designed to neutralise suppressor activity during *ex vivo* expansion if using PBMC as the source of T-cells.

In addition to Tregs, other cells of haematopoietic origin have been identified that can suppress immunity. Defects in maturation pathways of dendritic cells lead to the accumulation of immature myeloid cells (ImC) in most cancer patients to levels 5 times higher than that observed in normal healthy individuals (< 3%) [83]. ImC, like Tregs, when added to cul-

tures significantly inhibit antigen-specific T-cell proliferation. ImC can be induced to differentiate to mature DCs in the presence of all-*trans*-retinoic acid which simultaneously reduces their ability to inhibit T-cell function. It appears that the accumulation of ImC is a direct result of tumour presence since surgical removal of tumour can at least partially reverse these defects. The identification of suppressive haematopoietic T-cells in cancer patients is important and suggests that adoptive T-cells therapy, as well as tumour antigen-specific *ex vivo* expansion, can be improved through prior depletion of either Tregs or ImC.

6. Applications of imaging to immunotherapy

Recently developed biochemical imaging methods, in particular PET, play an increasing role in clinical oncology [84]. PET imaging may be helpful in the development of adoptive immunotherapy, in particular in three areas:

- monitoring tumour response to treatment
- tracking T-cell homing
- measuring the heterogeneity of antigen expression

The standard approach to measuring tumour response is to look for a decrease in tumour size as tumour cells die and the tumour mass shrinks [85]. However, size changes can lag cellular responses by weeks to months and for cytostatic therapies, tumour size may not change at all. It would therefore be logical to use biochemical imaging to look for evidence of tumour response earlier in the course of treatment. In this regard, PET imaging using 18-F fluorodeoxyglucose (FDG) has shown that tumour glucose metabolism can decline early in response to successful cytotoxic chemotherapy, well in advance of changes in tumour size [86,87]. For immunotherapy, because a local immune response at the tumour site may be energy requiring, it may be difficult to evaluate tumour response by FDG PET alone. Recent work with PET tracers of cellular proliferation, such as 11-C thymidine, has shown that measuring tumour cellular proliferation provides an early and quantitative estimate of tumour response to cytotoxic chemotherapy [87,88] and that the early changes in cellular proliferation in response to therapy are larger and more consistent than changes in glucose metabolism [87]. The combination of glucose metabolism and cellular proliferation measurements using PET, over the course of immunotherapy, will provide unique insights into the mechanisms and timing of the immune response *in vivo*.

To date, measuring the response of tumours to adoptive T-cell therapy has been limited to techniques that are restricted in their ability to quantify real time *in vivo* T-cell trafficking and homing. Indirect methods of monitoring therapy such as limiting dilution assays and ELISPOT can underestimate the number of antigen-specific T-cells at the tumour site, while more direct methods such as tetramer complexes are restricted to select MHC molecules. The radiolabelling of T-cells to allow *in vivo* trafficking offers promise in understanding

tumour-host interactions, T-cell expansion and T-cell homing during adoptive T-cell therapy. In addition to non-specific labelling of *ex vivo* expanded T-cells, molecular imaging approaches targeting the expression of specific genes using reporters designed to work with particular PET tracers [89] may be able to take advantage of T-cell markers specific for different T-cell subsets with unique functional phenotypes. Imaging the homing of T-cells to tumours and regional lymph nodes can provide new insight into tumour-host interactions. In the setting of adoptive T-cell therapy, these methods can elucidate the functional interaction of different T-cell subsets with tumour cells and other effector arms of the immune system.

One potential explanation for the lack of success with early trials of antigen specific T-cell therapy is the development of tumour antigen-loss variants. Tumours may escape recognition by adoptively transferred T-cells by downregulation of antigens resulting in a tumour that can evade detection and destruction. Measuring the heterogeneity of antigen expression using radiolabelled antibodies may help identify alterations in antigen expression as a cause of therapy failure. This can be done using the single-photon emitting isotope 131-I, as in the recently described approach which predicted Herceptin efficacy and cardiotoxicity [90]; or possibly with the positron-emitter, 124-I, to provide a more quantitative measure of antigen expression than can be achieved using [¹³¹I] and conventional radiotracer imaging.

The application of PET imaging technology to measuring tumour metabolism and proliferation, T-cell trafficking and antigen expression in adoptive T-cell therapy may yield insights into the mechanisms underlying functional adoptive immunotherapy and host-tumour interactions. Such studies are currently underway in the authors laboratory and others. Learning more about the functional interactions between T-cells and tumour cells will enable the development of more specific and long lasting responses to tumours and ultimately improve the efficacy of adoptive T-cell therapy.

7. Conclusion

Evidence from both mouse experiments and human clinical trials suggests that the most effective T-cell populations will be those that aim to reconstitute full T-cell immunity representing both major T-cell subsets, CTL and Th. In order to generate an adequate T-cell population, *ex vivo*, appropriate culture conditions must be established and may be different for each individual T-cell subset or antigen-presentation system. A number of cytokines have been cloned and produced that can have important effects in culture when used with IL-2. Furthermore, novel methods of antigen-presentation have been established to facilitate *ex vivo* expansion. Recent progress in molecular biology has led to the development of more efficient methods for cloning, altering and transfecting T-cells in order to improve important T-cell characteristics such as antigen-specificity and *in vivo* longevity. Finally, the implementation of *in vivo* imaging using PET should provide a greater

understanding of those characteristics that T-cells must possess in order to effectively home to and eradicate tumour.

Acknowledgements

This work was supported for Knutson by a fellowship from the Department of Defense Breast Cancer Program, for

Mankoff by NIH grants P01CA42045 and R01CA70264, for Almand by an NIH Basic Science Training Fellowship grant DC00018 and for Disis by a Cancer Treatment Research Foundation grant and NCI grants R01CA85374 and K24CA85218. The authors would like to thank Dr Janet Eary for helpful comments and Marsha Weese for expert editorial assistance.

Bibliography

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

- CHEEVER MA, CHEN W: Therapy with cultured T cells: principles revisited. *Immunol. Rev.* (1997) 157:177-194.
- A well-done overview of methods for *ex vivo* expansion and *in vivo* maintenance of tumour antigen-specific T-cells in a murine leukaemia model.**
- ROSKROW MA, SUZUKI N, GAN Y *et al.*: Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. *Blood* (1998) 91:2925-2934.
- ROONEY CM, SMITH CA, NG CY *et al.*: Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* (1995) 345:9-13.
- HESLOP HE, NG CY, LI C *et al.*: Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat. Med.* (1996) 2:551-555.
- SPRENT J, KISHIMOTO H: The thymus and central tolerance. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* (2001) 356:609-616.
- LECHLER R, CHAI JG, MARELLI-BERG F, LOMBARDI G: T-cell anergy and peripheral T-cell tolerance. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* (2001) 356:625-637.
- DUDLEY ME, WUNDERLICH J, NISHIMURA MI *et al.*: Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J. Immunother.* (2001) 24:363-373.
- ROSENBERG SA, LOTZE MT, MUUL LM *et al.*: A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N. Engl. J. Med.* (1987) 316:889-897.
- FISHER RI, COLTMAN CAJ, DOROSHOW JH *et al.*: Metastatic renal cancer treated with interleukin-2 and lymphokine-activated killer cells. A Phase II clinical trial. *Ann. Intern. Med.* (1988) 108:518-523.
- STEWART JA, BELINSON JL, MOORE AL *et al.*: Phase I trial of intraperitoneal recombinant interleukin-2/lymphokine-activated killer cells in patients with ovarian cancer. *Cancer Res.* (1990) 50:6302-6310.
- LUM LG: T cell-based immunotherapy for cancer: a virtual reality? *CA Cancer J. Clin.* (1999) 49:74-100, 65.
- BELLDEGRUN A, MUUL LM, ROSENBERG SA: Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity. *Cancer Res.* (1988) 48:206-214.
- RATTO GB, ZINO P, MIRABELLI S *et al.*: A randomized trial of adoptive immunotherapy with tumor-infiltrating lymphocytes and interleukin-2 versus standard therapy in the postoperative treatment of resected nonsmall cell lung carcinoma. *Cancer* (1996) 78:244-251.
- ROSENBERG SA, YANNELLI JR, YANG JC *et al.*: Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst.* (1994) 86:1159-1166.
- SCHWARTZENTRUBER DJ, HOM SS, DADMARZ R *et al.*: *In vitro* predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2. *J. Clin. Oncol.* (1994) 12:1475-1483.
- TO WC, WOOD BG, KRAUSS JC *et al.*: Systemic adoptive T-cell immunotherapy in recurrent and metastatic carcinoma of the head and neck: a Phase 1 study. *Arch. Otolaryngol. Head Neck Surg.* (2000) 126:1225-1231.
- PLAUTZ GE, MILLER DW, BARNETT GH *et al.*: T cell adoptive immunotherapy of newly diagnosed gliomas. *Clin. Cancer Res.* (2000) 6:2209-2218.
- ROONEY CM, ROSKROW MA, SUZUKI N, NG CY, BRENNER MK, HESLOP H: Treatment of relapsed Hodgkin's disease using EBV-specific cytotoxic T cells. *Ann. Oncol.* (1998) 9(Suppl. 5):S129-S132.
- WALTER EA, GREENBERG PD, GILBERT MJ *et al.*: Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N. Engl. J. Med.* (1995) 333:1038-1044.
- DISIS ML, KNUTSON KL, SCHIFFMAN K, RINN K, MCNEEL DG: Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. *Breast Cancer Res. Treat.* (2000) 62:245-252.
- KNUTSON KL, SCHIFFMAN K, DISIS ML: Immunization with a HER-2/neu helper peptide vaccine generates of HER-2/neu CD8 T cell immunity in cancer patients. *J. Clin. Invest.* (2001) 107:477-484.
- Demonstrates that late stage cancer patients with HER-2/neu positive tumours generate HER-2/neu-specific CD8 T-cell immunity following immunisation with a Th cell peptide vaccine of which each peptide contained nested HLA-A2 binding motifs. The resulting CD8 T-cells persisted for up to one year.**
- KNUTSON KL, CROSBY P, DISIS ML: Isolation of HER-2/neu (HER2) specific T cell clones from a cancer patient following immunization with a HER2 peptide vaccine. *Proc. Am. Assoc. Cancer Res.* (2000) 40:310-310. Abstract.
- KNUTSON KL, DISIS ML: Clonal diversity of the T cell repertoire responding to a dominant HLA-A2 epitope of HER-2/neu after active immunization. (Submitted) (2001).
- REDDISH M, MACLEAN GD, KOGANTY RR *et al.*: Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. *Int. J. Cancer* (1998) 76:817-823.

25. MATLOUBIAN M, CONCEPCION RJ, AHMED R: CD4⁺ T cells are required to sustain CD8⁺ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* (1994) 68:8056-8063.
26. ANDREASEN SO, CHRISTENSEN JE, MARKER O, THOMSEN AR: Role of CD40 ligand and CD28 in induction and maintenance of antiviral CD8⁺ effector T cell responses. *J. Immunol.* (2000) 164:3689-3697.
27. BATTEGAY M, MOSKOPHIDIS D, RAHEMTULLA A, HENGARTNER H, MAK TW, ZINKERNAGEL RM: Enhanced establishment of a virus carrier state in adult CD4⁺ T-cell-deficient mice. *J. Virol.* (1994) 68:4700-4704.
28. THOMSEN AR, JOHANSEN J, MARKER O, CHRISTENSEN JP: Exhaustion of CTL memory and recrudescence of viremia in lymphocytic choriomeningitis virus-infected MHC class II-deficient mice and B cell-deficient mice. *J. Immunol.* (1996) 157:3074-3080.
29. COHEN PA, PENG L, PLAUTZ GE, KIM JA, WENG DE, SHU S: CD4⁺ T cells in adoptive immunotherapy and the indirect mechanism of tumor rejection. *Crit. Rev. Immunol.* (2000) 20:17-56.
30. KAYAGAKI N, YAMAGUCHI N, NAKAYAMA M *et al.*: Involvement of TNF-related apoptosis-inducing ligand in human CD4⁺ T cell-mediated cytotoxicity. *J. Immunol.* (1999) 162:2639-2647.
31. ABE K, KURAKIN A, MOHSENI-MAYBODI M, KAY B, KHOSRAVI-FAR R: The complexity of TNF-related apoptosis-inducing ligand. *Ann. NY Acad. Sci.* (2000) 926:52-63.
32. DISIS ML, CHEEVER MA: HER-2/neu oncogenic protein: issues in vaccine development. *Crit. Rev. Immunol.* (1998) 18:37-45.
33. DISIS ML, GRABSTEIN KH, SLEATH PR, CHEEVER MA: Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin. Cancer Res.* (1999) 5:1289-1297.
34. GATELY MK, WILSON DE, WONG HL: Synergy between recombinant interleukin 2 (rIL 2) and IL 2-depleted lymphokine-containing supernatants in facilitating allogeneic human cytolytic T lymphocyte responses *in vitro*. *J. Immunol.* (1986) 136:1274-1282.
35. WOLF SE, TEMPLE PA, KOBAYASHI M *et al.*: Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.* (1991) 146:3074-3081.
36. GATELY MK, DESAI BB, WOLITZKY AG *et al.*: Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol.* (1991) 147:874-882.
37. UHEROVA P, CONNICK E, MAWHINNEY S, SCHLICHTEMEIER R, SCHOOLEY RT, KURITZKES DR: *In vitro* effect of interleukin-12 on antigen-specific lymphocyte proliferative responses from persons infected with human immunodeficiency virus Type 1. *J. Infect. Dis.* (1996) 174:483-489.
38. MCFARLAND EJ, HARDING PA, MAWHINNEY S, SCHOOLEY RT, KURITZKES DR: *In vitro* effects of IL-12 on HIV-1-specific CTL lines from HIV-1-infected children. *J. Immunol.* (1998) 161:513-519.
39. TRINCHIERI G: Function and clinical use of interleukin-12. *Curr. Opin. Hematol.* (1997) 4:59-66.
40. DISIS ML, GOOLEY TA, RINN K *et al.*: Generation of T Cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. *J. Clin. Oncol.* (in press) (2001).
41. KNUTSON KL, DISIS ML: IL-12 enhances the function and number of tumor antigen-specific Th1 lymphocytes during *ex vivo* expansion. (Submitted) (2001).
42. TAN JT, DUDL E, LEROY E *et al.*: IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. USA* (2001) 98:8732-8737.
43. SCHULTZE JL, SEAMON MJ, MICHALAK S, GRIBBEN JG, NADLER LM: Autologous tumor infiltrating T cells cytotoxic for follicular lymphoma cells can be expanded *in vitro*. *Blood* (1997) 89:3806-3816.
44. TSAI V, KAWASHIMA I, KEOGH E, DALY K, SETTE A, CELIS E: *In vitro* immunization and expansion of antigen-specific cytotoxic T lymphocytes for adoptive immunotherapy using peptide-pulsed dendritic cells. *Crit. Rev. Immunol.* (1998) 18:65-75.
45. GRABSTEIN KH, EISENMAN J, SHANEBECK K *et al.*: Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* (1994) 264:965-968.
46. MA A, BOONE DL, LODOLCE JP: The pleiotropic functions of interleukin 15: not so interleukin 2-like after all. *J. Exp. Med.* (2000) 191:753-756.
47. WALDMANN TA, DUBOIS S, TAGAYA Y: Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. *Immunity* (2001) 14:105-110.
48. WENINGER W, CROWLEY MA, MANJUNATH N, VON ANDRIAN UH: Migratory properties of naive, effector, and memory CD8(+) T cells. *J. Exp. Med.* (2001) 194:953-966.
49. DYBUL M, MERCIER G, BELSON M *et al.*: CD40 ligand trimer and IL-12 enhance peripheral blood mononuclear cells and CD4⁺ T cell proliferation and production of IFN-gamma in response to p24 antigen in HIV-infected individuals: potential contribution of anergy to HIV-specific unresponsiveness. *J. Immunol.* (2000) 165:1685-1691.
50. GREWAL IS, FLAVELL RA: CD40 and CD154 in cell-mediated immunity. *Ann. Rev. Immunol.* (1998) 16:111-135.
51. KEDL RM, JORDAN M, POTTER T, KAPPLER J, MARRACK P, DOW S: CD40 stimulation accelerates deletion of tumor-specific CD8(+) T cells in the absence of tumor-antigen vaccination. *Proc. Natl. Acad. Sci. USA* (2001) 98:10811-10816.
52. TERHEYDEN P, STRATEN P, BROCKER EB, KAMPGEN E, BECKER JC: CD40-ligated dendritic cells effectively expand melanoma-specific CD8⁺ CTLs and CD4⁺ IFN-gamma-producing T cells from tumor-infiltrating lymphocytes. *J. Immunol.* (2000) 164:6633-6639.
53. REILLY RT, MACHIELS JP, EMENS LA *et al.*: The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res.* (2000) 61:880-883.
54. BANCHEREAU J, STEINMAN RM: Dendritic cells and the control of immunity. *Nature* (1998) 392:245-252.
55. KUROKAWA T, OELKE M, MACKENSEN A: Induction and clonal expansion of tumor-specific cytotoxic T lymphocytes from renal cell carcinoma patients after stimulation with autologous

- dendritic cells loaded with tumor cells. *Int. J. Cancer* (2001) 91:749-756.
56. YEE C, SAVAGE PA, LEE PP, DAVIS MM, GREENBERG PD: Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *J. Immunol.* (1999) 162:2227-2234.
 - Describes the utility of using tetramers to isolate high avidity, antigen-specific peptide-specific T-cells for *ex vivo* clonal expansion. Discusses the application to adoptive T-cell therapy of melanoma.
 57. HEISER A, MAURICE MA, YANCEY DR *et al.*: Induction of polyclonal prostate cancer-specific CTL using dendritic cells transfected with amplified tumor RNA. *J. Immunol.* (2000) 166:2953-2960.
 58. HEISER A, MAURICE MA, YANCEY DR, COLEMAN DM, DAHM P, VIEWEG J: Human dendritic cells transfected with renal tumor RNA stimulate polyclonal T-cell responses against antigens expressed by primary and metastatic tumors. *Cancer Res.* (2001) 61:3388-3393.
 59. MEYER ZB, METZGER J, HERMANN C, NICKLISCH N, PESCHEL C, BERNHARD H: The generation of both T killer and Th cell clones specific for the tumor-associated antigen her2 using retrovirally transduced dendritic cells. *J. Immunol.* (2001) 167:1712-1719.
 - Demonstrates that HER-2/neu-specific T-cells, both helper and CTL, can be expanded selectively in culture using autologous HER-2/neu-expressing DCs derived from CD34 progenitor cells. Discusses potential application to adoptive T-cell immunotherapy and the generation of high affinity antigen-specific T-cells.
 60. KE Y, MA H, KAPP JA: Antigen is required for the activation of effector activities, whereas interleukin 2 is required for the maintenance of memory in ovalbumin-specific, CD8+ cytotoxic T lymphocytes. *J. Exp. Med.* (1998) 187:49-57.
 61. MORRIS A, VETTO JT, RAMSTAD T *et al.*: Induction of anti-mammary cancer immunity by engaging the OX-40 receptor *in vivo*. *Breast Cancer Res. Treat.* (2001) 67:71-80.
 62. WEINBERG AD, RIVERA MM, PRELL R *et al.*: Engagement of the OX-40 receptor *in vivo* enhances antitumor immunity. *J. Immunol.* (2000) 164:2160-2169.
 63. KJAERGAARD A, WEINBERG A, SHU S: Characteristics of antibody-mediated OX-40R ligation in augmentation of adoptive T-cell immunotherapy of advanced tumor. *Proc. Am. Assn. Canc. Res.* (2001) 42:328. Abstract.
 64. GREENBERG PD, FINCH RJ, GAVIN MA *et al.*: Genetic modification of T-cell clones for therapy of human viral and malignant diseases. *Cancer J. Sci. Am.* (1998) 4 (Suppl. 1):S100-S105.
 - Underscores the potential application and importance of genetically modifying T-cells for improving function and extending life.
 65. STEINMAN L: Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* (1996) 85:299-302.
 66. TISCH R, MCDEVITT H: Insulin-dependent diabetes mellitus. *Cell* (1996) 85:291-297.
 67. TUOHY VK, MATHISEN PM: T-cell design: optimizing the therapeutic potential of autoreactive T cells by genetic modification. *Res. Immunol.* (1998) 149:834-842.
 - The autoreactive T-cells of diabetes and EAE can be genetically altered to deliver immunosuppressive molecules to sites of inflammation in order to suppress disease symptoms.
 68. SHAW MK, LORENS JB, DHAWAN A *et al.*: Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. *J. Exp. Med.* (1997) 185:1711-1714.
 69. MORITANI M, YOSHIMOTO K, II S *et al.*: Prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes. A gene therapy model for autoimmune diabetes. *J. Clin. Invest.* (1996) 98:1851-1859.
 70. EVANS LS, WITTE PR, FELDHAUS AL *et al.*: Expression of chimeric granulocyte-macrophage colony-stimulating factor/interleukin 2 receptors in human cytotoxic T lymphocyte clones results in granulocyte-macrophage colony-stimulating factor-dependent growth. *Hum. Gene Ther.* (1999) 10:1941-1951.
 71. CALOGERO A, HOSPERS GA, KRUSE KM *et al.*: Retargeting of a T cell line by anti MAGE-3/HLA-A2 alpha beta TCR gene transfer. *Anti-Cancer Res.* (2000) 20:1793-1799.
 72. DALY T, ROYAL RE, KERSHAW MH *et al.*: Recognition of human colon cancer by T cells transduced with a chimeric receptor gene. *Cancer Gene Ther.* (2000) 7:284-291.
 73. ROSSIG C, NUCHTERN JG, ROONEY CM, BRENNER MK: Targeting of tumor cells with EBV-specific human T lymphocytes transduced to express tumor-specific chimeric T cell receptor genes. *Proc. Am. Assoc. Cancer Res.* (2001) 42:845. Abstract.
 74. JONULEIT H, SCHMITT E, STASSEN M, TUETTENBERG A, KNOP J, ENK AH: Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* (2001) 193:1285-1294.
 75. LEVINGS MK, SANGREGORIO R, RONCAROLO MG: Human CD25(+)CD4(+) regulatory cells suppress naive and memory T cell proliferation and can be expanded *in vitro* without loss of function. *J. Exp. Med.* (2001) 193:1295-1302.
 76. DIECKMANN D, PLOTTNER H, BERCHTOLD S, BERGER T, SCHULER G: *Ex vivo* isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J. Exp. Med.* (2001) 193:1303-1310.
 77. STEPHENS LA, MOTTET C, MASON D, POWRIE F: Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity *in vitro*. *Eur. J. Immunol.* (2001) 31:1247-1254.
 78. WOO EY, CHU CS, GOLETT TJ *et al.*: Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res.* (2001) 61:4766-4772.
 79. JORDAN MS, BOESTEANU A, REED AJ *et al.*: Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* (2001) 2:301-306.
 80. CEDERBOM L, HALL H, IVARS F: CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur. J. Immunol.* (2000) 30:1538-1543.
 81. ONIZUKA S, TAWARA I, SHIMIZU J, SAKAGUCHI S, FUJITA T, NAKAYAMA E: Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res.* (1999) 59:3128-3133.
 82. SHIMIZU J, YAMAZAKI S, SAKAGUCHI S: Induction of tumor immunity by removing CD25+CD4+ T

- cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* (1999) 163:5211-5218.
83. ALMAND B, CLARK JI, NIKITINA E *et al.*: Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J. Immunol.* (2001) 166:678-689.
84. EARY JF: Nuclear medicine in cancer diagnosis. *Lancet* (1999) 354:853-857.
85. HUSBAND JE: Monitoring tumour response. *Eur. Radiol.* (1996) 6:775-785.
86. WAHL RL, QUINT LE, CIESLAK RD, AISEN AM, KOEPPE RA, MEYER CR: 'Anatometabolic' tumor imaging: fusion of FDG PET with CT or MRI to localize foci of increased activity. *J. Nucl. Med.* (1993) 34:1190-1197.
87. SHIELDS AF, MANKOFF DA, LINK JM *et al.*: Carbon-11-thymidine and FDG to measure therapy response. *J. Nucl. Med.* (1998) 39:1757-1762.
88. MANKOFF DA, DEHDASHTI F, SHIELDS AF: Characterizing tumors using metabolic imaging: PET imaging of cellular proliferation and steroid receptors. *Neoplasia* (2000) 2:71-88.
89. GAMBHIR SS, HERSCHMAN HR, CHERRY SR *et al.*: Imaging transgene expression with radionuclide imaging technologies. *Neoplasia* (2000) 2:118-138.
90. BEHR TM, BEHE M, WORMANN B: Trastuzumab and breast cancer. *N. Engl. J. Med.* (2001) 345:995-996.

Affiliation

Keith L Knutson^{1†}, Bond Almand¹, David A Mankoff², Kathy Schiffman¹ & Mary L Disis¹

[†]Author for correspondence

¹Division of Oncology, University of Washington, 1959 NE Pacific Street, HSB BB1321, Box 356527, Seattle, WA 98195-6527, USA

Tel.: +1 206 221 5417; Fax: +1 206 685 3128;

E-mail: kknutson@u.washington.edu

²Division of Nuclear Medicine, University of Washington, Seattle, WA 98195, USA

APPENDIX 5

CHAPTER 17

Immunotherapy for breast cancer

Keith L. Knutson, Michael R. Bishop, Kathy Schiffman and Mary L. Disis

1. Introduction

Immune-based therapies for the treatment of breast cancer are now being designed to circumvent specific biologic problems that have been identified in the native immune response to breast cancer. Advances in basic immunology over the past decade have changed the scope and application of breast cancer immunotherapy. Perhaps the most important advance is the understanding that human tumors are immunogenic and the identification of tumor-specific antigens. In fact, hundreds of immunogenic proteins have been identified in patients with cancer using high throughput technologies such as serological identification of antigens by recombinant expression cloning (SEREX). More importantly, we have begun to identify proteins that are immunogenic in cancer whose function may be a causal component in the malignant transformation. Thus, immune-based therapies targeting these biologically relevant antigens may result in the eradication of tumor cell clones that drive the initiation of malignancy.

The roles of the immune and tumor microenvironments in breast cancer greatly influence our approach to the design of immunotherapies. Stimulation of both cytotoxic T cell (CTL) and CD4 'helper' T cell immunity is critically important for an effective antitumor response. In addition, cells of the innate immune system such as nat-

ural killer (NK) cells are likely to play an important role in a balanced antitumor response. Cytokines and chemokines can both inhibit and stimulate immune responses and tumor growth. Moreover, the identification of the dendritic cell (DC) and the understanding of its role in initiating immunity to cancer has not only led to novel therapies for the treatment of breast cancer, but has focused attention on the major role of the antigen-presenting cell (APC) in initiation or dampening of the tumor-specific immune response. We now know that the tumor microenvironment is dynamic. Molecular dissection of the evolution of the immune response occurring against autologous tumors in patients with advanced stage melanoma has demonstrated that the immune system is quite functional even in patients with significant existing tumor burden. In fact, stimulating immunity in patients with measurable disease may have beneficial effects in immune augmentation. Apoptosis or necrosis of tumor cells may be an efficient method of antigen presentation for initiation of immune responses. Likewise, the presence of antigen during the evolution of the immune response may be instrumental in generating T cell clonal expansion and epitope spreading highlighting the occurrence of natural antigen-processing and the generation of endogenous tumor-specific immunity.

Finally, immune-based therapies are being applied clinically in different ways, both for treat-

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ment and prevention. Vaccines targeting tumor antigens are increasingly being viewed and studied as chemopreventive agents designed to protect against cancer relapse or development. Established disease is being treated with immune-based therapies, such as adoptive immunotherapy or infusion of T cells allogeneic to host tumor cells therapies, designed to maximize T cell precursor frequencies. Thus, investigations of the immune system at both the bench and bedside have led to the development of rationally based immunotherapies for the prevention and treatment of breast cancer. Therapeutic approaches are now targeted to specific alterations in the evolution of the breast cancer-specific immune response.

2. Breast cancer tumor antigens

Recent studies by several groups have identified 'self' antigens, expressed on tumor cells, as tumor antigens [1,2]. These proteins are not mutated in any way, but are clearly immunogenic in patients with cancer and have been shown to generate both antibody and CTL responses in humans [1,3,4]. Many of these proteins are present at much higher concentrations in malignant cells than in the normal cells with which they are associated [5]. Gene amplification results in overexpression of normal cellular proteins in cancer and is an etiologic factor in the malignant transformation of many solid tumors. Overexpressed oncoproteins are not mutated and are distinct from their normal counterparts only by virtue of their greater concentration in cancer cells. Intuitively, these proteins would not be considered potential tumor antigens as patients should be tolerant to self-proteins. The recent finding, that many tumor antigens are self-proteins, has resulted in a 'paradigm shift' [6]. The new paradigm includes self-proteins as tumor antigens and tolerance induction as a possible mechanism of immune escape.

A multitude of self-tumor antigens are present in breast cancer including, CEA, MUC-1, a variety of glycoproteins, p53, and MAGE-3 to name a few. Of interest is the identification of im-

munogenic proteins in breast cancer that are also important cell regulatory proteins. By targeting the immune system to proteins that either initiate or propagate the malignant phenotype immune-based therapies have the potential to eradicate the malignant clone. An example of two defined tumor antigens that are involved in the carcinogenic pathway of breast cancer are the HER-2/neu oncogenic protein and telomerase.

One of the most extensively described tumor antigens in breast cancer is the HER-2/neu oncogenic protein. HER-2/neu, is a member of the epidermal growth factor receptor family and is a growth factor receptor [7,8]. HER-2/neu is a transmembrane protein that consists of a cysteine-rich extracellular domain that functions in ligand binding and a cytoplasmic domain with kinase activity. In humans, the HER-2/neu protein is expressed during fetal development [9]. In adults, the protein is weakly detectable in the epithelial cells of many normal tissues by immunohistochemical staining. The HER-2/neu gene is present in normal cells as a single copy [9]. Amplification of the gene and/or overexpression of the associated protein have been identified in many human cancers such as breast, ovarian, prostate, non-small cell lung cancer and colon cancer and is associated with a poor prognosis. The HER-2/neu oncogenic protein is also a tumor antigen. Patients with different tumor types that overexpress the HER-2/neu protein can have both antibody [10] and T cell immunity directed against HER-2/neu [11]. Existent immunity to HER-2/neu in humans was initially described in patients with breast cancer [3].

Telomerase is a ribonucleoprotein enzyme. Normally telomerase maintains the telomeric ends of chromosomes protecting them from degradation. Non-malignant cells do not express telomerase, but malignant cells have increased telomerase activity presumably resulting in immortalization of the cell. The telomeric catalytic subunit (hTERT) is the rate-limiting component of the enzyme and expression of hTERT correlates best with telomerase activity [12]. Although hTERT is

a self-protein, CTL specific for peptides derived from hTERT can lyse hTERT expressing tumor cells in an HLA-A2-restricted fashion. The ability to recognize hTERT is within the realm of the T cell repertoire [12]. The definition of biologically important and ubiquitous proteins as tumor antigens in breast cancer alters our concept of what characteristics of a protein make it immunogenic. No longer are tumor antigens defined by unique mutations. The problem currently facing tumor immunologists is how to harness immunity to 'self' for cancer therapeutics. Both the immune and tumor microenvironments play an important role in regulating tumor-specific immunity.

3. Role of the immune microenvironment in regulating breast cancer immunity

Elucidation of breast cancer-specific immunity in animal models has demonstrated that an effective antitumor immune response will likely consist of complex interactions among multiple cellular and soluble immune effectors. Effector cells other than CTL that may induce an antitumor immune response include CD4⁺ T cells, NK cells, NKT cells, DC, and other 'non-professional' APC such as macrophages. Furthermore, many chemokines and cytokines have been characterized that can have profound effects on immune effector cells as well as tumor cells.

CD8 or cytotoxic T cells (CTL) have been thought to be major mediators of antitumor immunity because they exhibit direct killing of tumor cells in an MHC class I-restricted fashion. In studies in breast cancer patients, CTL established *in vitro* demonstrate significant levels of cytolytic activity against known breast cancer antigens such as MUC-1 and HER-2/neu [4,13]. In fact, similar to analyses performed in melanoma, recent studies evaluating the presence of class I molecules in breast cancer, have shown that class I can be down-regulated presumably due to immunosélection [14]. Therefore, CTL specific for breast cancer antigens do exist naturally *in vivo* in patients with breast cancer and may have a biologic

effect on the tumor, but clearly are not sufficient in eradicating cancer. One problem with the pre-existent CTL response may be a lack of magnitude. An evaluation of immunity against the HER-2/neu antigen in patients with advanced stage breast cancer suggests that CTL precursor frequencies elicited by exposure to tumor are low, less than 1:100,000 of circulating T cells [15]. Direct modulation of the CTL immune effector arm is thought to be key to a successful antitumor response. However, recent developments suggest that CTL alone cannot constitute a complete antitumor response and that additional immune effectors such as CD4⁺T cells are needed for completing the appropriate immune milieu.

The CD4⁺ T cell is critical in controlling the activation and persistence of the immune response against viral infections [16]. The interplay between CD8⁺ and CD4⁺ T cells in eradicating breast cancer has recently been evaluated in the neu transgenic (neuTg) mouse. neuTg mice are transgenic for rat neu under control of the MMTV promoter and develop breast cancers similar to those that occur in humans [17]. Also, like in humans, expression as a transgene confers immunologic tolerance to neu. The murine cancers generally start as hyperplasia, progress to ductal carcinomas *in situ*, and finally to frank interductal carcinomas [17]. Biologically relevant animal models, such as this one, can significantly aid in the definition of the functional components of the breast cancer-specific immune response as well as identify methods of overcoming tolerance. Although tolerant, neuTg mice can be vaccinated against neu and develop both neu-specific T cells and neu-specific IgG [18]. The resulting immune response, after active immunization, can delay the onset of the spontaneous neu-mediated tumors. In addition, depletion studies demonstrated that not only are CD8⁺ T cells required for therapeutic efficacy, but also CD4⁺ T cells. In fact, CD4⁺ T cell-depleted mice showed faster tumor development than CD8⁺-depleted mice suggesting a dominant role for neu-specific CD4⁺ T cells in eradicating the murine breast cancers.

Furthermore, to eradicate neu transgenic tumor implanted in SCID mice, both an infusion of neu-specific CTL and neu-specific IgG were needed [19]. The importance of CD4⁺ T cells in the evolution of the tumor-specific immune response is underscored by recent studies demonstrating that CD4⁺ T cells can initiate a de novo CTL response. Infusion of a tumor antigen-specific Th1 CD4⁺ T cell clone resulted in the development of a CD8⁺ antitumor immune response, presumably due to CD4⁺ T cells secreting cytokines appropriate to enhance the function of local APC to cross-present tumor antigen to endogenous naïve CD8⁺ T cells [20]. Once a CD4⁺ T cell becomes activated a number of important events take place to influence the immune microenvironment. In addition to initiation of a CTL response, CD4⁺ T cells can also produce cytokines that attract DC, macrophages, and eosinophils to promote an inflammatory environment [21]. As a result of establishing the necessity of CD4⁺ T cell help in the immune response, much interest has focused on the identification of MHC class II binding epitopes of well-known human breast tumor antigens such as HER-2/neu [22] and NY-ESO-1 [23,24].

Although the antigen-specific T lymphocyte immune response is most often the focus of experimental and clinical investigation, cellular effectors of the innate immune system, such as NK and NKT cells also can induce an antitumor response. NK cells can kill tumors through a non-MHC-restricted mechanism. Newly defined NKT cells can specifically lyse tumor cells bearing glycolipid antigens. Inflammatory mediators, such as IL-12, may exert their antitumor effects through activation of NK cells. A murine model of metastatic breast cancer was developed to evaluate the therapeutic efficacy of administration of IL-12 in an adenoviral vector directly into liver lesions. Lesions regressed with treatment in almost all animals and complete tumor rejection occurred in 40% [25]. Cell subset depletion studies revealed that the antitumor effect seen with IL-12 was mediated significantly by NK cells. Although NK cells can possess direct antitumor

cytolytic activity *in vitro* the role for the NK cell in mediating tumor rejection *in vivo* is still unclear. In addition to their ability to directly kill tumor, NK cells can also release a variety of type I cytokines such as IFN γ and TNF α which influence development of the adaptive immune response.

A newly identified cell of a novel lymphoid lineage, the V α 14 NKT cell, introduces an additional effector cell that may be important in an antitumor immune response. The NKT cell expresses typical NK cell surface receptors and a semi-invariant T cell receptor (TCR) encoded by V α 14 and J α 281 [26]. The NKT cell is activated through recognition of glycolipid antigens in association with CD1b, a MHC-like molecule present on APC. NKT cells secrete predominantly IL-4 and IFN γ and may be important in regulating the Th1/Th2 cytokine phenotype during an immune response [26]. Recently, Nishimura and colleagues have shown that CD1d⁺ DC presentation of glycolipids to NKT cells can result in the generation of lymphoma-specific CTL suggesting an important interface between the innate and adaptive antitumor immune response [27]. Glycolipids, such as ceramides and gangliosides are some of the most commonly studied breast cancer antigens [28,29]. Indeed, glycosylation of glycoproteins and glycolipids is a molecular change that often accompanies the malignant transformation of a breast cell [30]. In addition, CD1⁺ DC are often observed in breast cancer biopsies [30]. Therapeutic infusion of NKT cells may be uniquely suited for the treatment of breast cancer.

The cellular immune response can not be elicited without effective APC processing and presentation of tumor proteins to T cells. DC are potent APC and are important in initiating a T cell response. DC usually exist in peripheral tissue in an immature state awaiting encounter with antigen. Immature DC are proficient at antigen uptake and processing but have no capacity to activate T cells. Following exposure to antigen, DC migrate to regional draining lymph nodes (DLN) and it is during migration that DC at-

tain the ability to present antigen, i.e. undergo maturation. Antigen-specific T lymphocytes are then stimulated by direct contact with the DC. Although not well described for breast cancer, most tumors fail to express MHC class II and are, therefore, not direct targets of CD4⁺ T cells. The tumor cells would be immunogenic by other means such as uptake of the tumor cell debris by APC and 'cross-priming' [31]. The uptake, processing, and presentation of exogenously derived tumor antigens by DC, therefore, is important for activation of CD4⁺ T cells. DC can acquire antigen by multiple means including, endocytosis of soluble protein and phagocytosis of tumor-derived exosomes and apoptotic bodies [32,33]. Recent investigations have demonstrated that DC are associated with breast cancer tissue. Studies by Bell and colleagues have revealed that the maturation state of the DC differs with proximity to the breast cancer lesion [34]. Immature DC reside within the tumors, while mature DC are located in peritumoral areas. The intratumoral localization of immature DC suggests that apoptosis-inducing or tumor necrotizing agents may be effective in treating breast cancer as this may result in the cross-presentation of tumor-specific antigen to CD4⁺T cells. Indeed, Candido and colleagues have recently observed that the level of tumor cell apoptosis is positively correlated to the effectiveness of DC-based therapy of murine MT-901 breast carcinomas [35].

Cytokines released by both T cells and other cells involved in the inflammatory response greatly influence the immune microenvironment. Traditionally, T cell responses are categorized into a type I, e.g. TNF α -, IFN γ -secreting or type II, e.g. IL-4-, IL-10- secreting phenotype. Type I cytokine T cell responses are typically associated with the development of cell-mediated immune response while type II cytokines lead to the development of a humoral response and down-modulation of CTL. The interaction between cytokines and the stimulation of a T cell response is complex. As an example, early studies demonstrating that IFN γ could activate CTL,

macrophages, and NK cells as well as up-regulate the level of MHC on a variety of immune effectors suggested that this cytokine is a major mediator of the antitumor immune response [36–39]. More recent investigations in IFN γ ^{-/-} mice, however, demonstrate that the function of IFN γ in mediating an antitumor immune response is complex. IFN γ ^{-/-} mice were immunized with syngeneic tumor cells. Spleen cells from immunized mice demonstrated antitumor activity, but when mice were challenged with viable tumor, they were not protected. T cell infiltration of tumor did not occur in the IFN γ ^{-/-} mice demonstrating that IFN γ was necessary for migration of both CD4⁺ and CTL to tumor sites [40]. Thus, a major influence of IFN γ at the tumor site may be as a chemoattractant.

NK cells are also targets of cytokines and chemokines as recently described in murine breast cancer models. It is well known that IL-12 can directly mediate tumor killing. Many mechanisms have been described for the effects of IL-12 including increasing the local production of IFN γ and the inhibition of angiogenesis [41]. IL-12 is also known to directly stimulate NK cells [25]. NK cells can also be activated by the chemokine CK β -11 to kill breast cancer cells [42]. CK β -11 functions as a chemoattractant for many immune effector cells including T cells, B cells, DC, and NK cells [43] [44,45]. In a study by Braun and colleagues, CK β -11 was transfected and expressed intratumorally in murine breast cancer. The resultant tumor rejection involved both NK and T lymphocytes.

Recent studies have focused on the *in vivo* modulation of APC function by cytokines which can act at many different levels including APC differentiation [46], lifespan [47], migration [43], and antigen presentation [48]. Several cytokines are known to promote dendropoiesis and include IL-12 and Flt3 ligand, both of which have been shown to mediate tumor regression *in vivo* [49,50]. While the administration of either IL-12 or Flt-3 ligand increases the total number of circulating DC, IL-12 promotes myeloid den-

dropoiesis exclusively while Flt3 ligand promotes both myeloid and lymphoid dendropoiesis [46]. In a study by Esche and colleagues, the addition of both IL-12 and Flt3 ligand synergized the expression of MHC class II suggesting that antigen presentation may be enhanced with combination cytokine treatment. Increasing the numbers of DC in the draining lymph nodes (DLN) can potentiate immune responses. DC die rapidly upon migration to the DLN, limiting the time of antigen exposure and the resulting immune response. A newly discovered TNF-related cytokine, TNF-related activation-induced cytokine (TRANCE) has been shown to enhance the lifespan of *ex vivo* manipulated DC [47]. By increasing the lifespan of antigen-presenting DC in the DLN, T cell responses to antigen were substantially increased [47]. Cytokines can down-regulate immune responses as well. As an example, lysosomal proteases of the cathepsin family are involved in processing of antigen in the context of MHC class II. IFN γ modulates cathepsin activity in cells [51]. Fieberger and colleagues recently examined the effects of proinflammatory and anti-inflammatory cytokines on the activities of cathepsin S and cathepsin B in human DC [48]. Treatment of DC with the proinflammatory cytokines TNF α and IL-1 β leads to a rapid increase in the levels of DC cathepsin while the anti-inflammatory cytokine IL-10 renders the DC incapable of up-regulation of cathepsin. Coincidentally, IL-10 also reduces the number of MHC class II antigen complexes on the cell surface. Thus, cytokines can function to enhance or dampen the breast cancer-specific immune response *in vivo*.

4. Role of the tumor microenvironment in regulating breast cancer immunity

Human tumors can suppress the development of an immune response. However, even in advanced stage cancer immunosuppression in patients may not be complete. In addition to the remarkable plasticity and ability to change phenotype, tumors can evade immune recognition by several

mechanisms including tolerance, ignorance, and inducing T cell dysfunction and T cell death.

Tumor-specific immunity developing in a patient with advanced stage cancer is well documented. Coulie and colleagues recently published a 15-year chronology of immunologic studies of a melanoma patient with multiple recurrences but with a favorable clinical evolution due to immunologic intervention, both natural and pharmacological [52]. The findings provided key *in vivo* evidence that the generation of an immune response can potentially result in the breakthrough of unrecognized variant tumor cells. The patient initially presented with a primary melanoma that was surgically removed. Following three relapses, a cell line, MEL A was established from a subcutaneous metastases and the patient underwent repeated vaccinations with the cell line and was disease-free for 4 years. This disease-free interval was associated with strong antitumor CTL response. CTL clones isolated from peripheral blood revealed several antigens produced by MEL A cells could be recognized in association with several HLA alleles. Eventually the patient relapsed with a tumor that had lost a complete HLA haplotype (A28-B44-Cw7). However, the original antigens were still expressed on the tumor. All of the CTL clones that were isolated before the relapse were A28- and B44-restricted. The development of escape variants has yet to be described in breast cancer, but as antigen-specific immune-based therapies are clinically tested evaluations of both antigen and immunostimulatory molecules on relapsed disease should be performed. Studies such as this one demonstrate that cancer patients can develop functional immune responses but that tumors can evade restricted immune responses.

Tolerance to self-tumor antigens is a major obstacle to overcome in the development of significant tumor-specific immunity. Tolerance can be mediated centrally and peripherally. Central tolerance occurs when antigens are expressed intrathymically resulting in deletion of the self-reactive T cells during early T cell differentiation [53]. Central tolerance is thought to delete pri-

marily high-affinity T cells that recognize dominant peptide epitopes, while lower-affinity T cells and those recognizing subdominant epitopes are allowed to escape into the periphery. Central tolerance leads to irreversible loss of autoantigen-reactive T cells by inducing apoptosis and it is only those autoreactive T cells that escape thymic deletion that are of importance clinically. The T cells that avoid thymic clearance however are often tolerant or ignorant of tissues expressing the target antigens. As tumor antigen-specific T cells can be found in patients with cancer, it is clear that the ability to recognize cancer antigens is within the realm of the T cell repertoire and that central tolerance is not complete. Peripheral tolerance occurs extrathymically and is mediated by both deletional and non-deletional mechanisms [54]. The T cells that have been tolerized by non-deletional methods are the focus of immune-based antitumor therapies. A major non-deletional mechanism of peripheral tolerance is anergy induction. Anergy induction of antigen-specific T cells occurs by multiple mechanisms. The best understood mechanism of T cell anergy induction is TCR signaling in the absence of co-stimulation. Activation of T cells is thought to involve at least two independent signals, one mediated through the TCR:MHC complex and another through any of a variety of co-stimulatory receptors on T cells including CD28. Tumor cells, including breast cancer, do not typically express receptors (e.g. CD80) to these co-stimulatory molecules and may be responsible for anergy induction. In fact, recent reports demonstrate that even APC in the peripheral blood of early stage breast cancer patients demonstrate decreased expression of CD80 and CD86 [55]. Anergic T cells when rechallenged with peptide-pulsed APC fail to proliferate and have reduced cytokine release. Indeed, transfection of CD80 into many tumors, including breast cancer, can restore immune recognition and cause growth arrest [56].

The recovery of anergic T cells and the reversal of tolerance is critical to successful development of immune-based strategies for the treatment of

breast cancer. Although it is generally presumed that self-reactive T cells are low-affinity by virtue of tolerizing processes, recent evidence by Ohlen and colleagues suggest that higher-affinity peripherally tolerized tumor-antigen-specific T cells can be recovered following multiple rounds of in vitro stimulation. Studies demonstrated that gag-specific T cells could be isolated from mice transgenically engineered to express the Friend murine leukemia virus protein, gag, within hepatocytes [57]. The gag-specific T cells, after several in vitro stimulations with repeated IL-2 administration, displayed CTL activity and were comparable in affinity to the gag-specific T cells isolated from the parental mouse, C57BL/6, in which gag is a foreign antigen. These findings suggest the potential for circumvention of tolerance and the recovery of high-affinity tumor-specific T cells using ex vivo techniques.

Cytokines present within the tumor microenvironment can also promote T cell anergy. The immunoregulatory cytokine, IL-10 has previously been shown to induce a state of profound non-responsiveness to melanoma and alloreactive T cells [58,59]. IL-10 acts directly on T cells during activation through CD28 and can be reversed with exogenous IL-2 [60]. Molecularly, IL-10 inhibits tyrosine phosphorylation of CD28 and association of PI3-kinase to CD28. These recent findings suggest that the systemic and locally elevated levels of IL-10 often found in cancer patients may further contribute to tolerance to tumor-associated antigens. Little is known of the role of IL-10 in suppressing immunity to breast cancer; however, IL-10 levels are elevated in pancreatic [61] and melanoma [62] cancer patients and elevated levels of IL-10 correlate with poor survival [62].

Tumors can also evade immune recognition by immunologic ignorance, which reflects the inability of naïve antigen-specific T cells to recognize tumor tissue. Ochsenbein and colleagues studied immune surveillance against solid tumors and observed that transplantation of tumor pieces into mice grew readily. However, if the same tumor cells were injected as a single-cell suspension,

?#2

?#3

?#4

a protective cytolytic response was observed. It was observed that tumor growth correlated with failure of tumor cells to reach the draining lymph nodes and the absence of primed cytotoxic T cells [63]. Ignorance to antigen-bearing tumor is also observed in the OT-1 mice which are transgenic for OVA-specific T cells. Adoptive transfer of naïve OT-1 cells into OVA-expressing tumor-bearing mice does not inhibit tumor growth even though OT-1 cells are able to lyse OVA-tumors *in vitro*. However, if the OT-1 cells were pre-activated prior to transfer, tumor protection was observed. Additionally, mice vaccinated with OVA-pulsed APC (i.e. cross-priming) could activate naïve OT-1 cells, which could protect against tumor. These results demonstrate that ignorance plays an important role in tumor evasion and that therapeutic intervention to activate tumor-specific T cells is necessary to overcome ignorance. Thus, while some tumors may anergize T cells, others may avoid immune recognition through ignorance [64].

Tumors can induce T cell dysfunction and even death. The CD95/CD95L (Fas/FasL) system plays an important role in limiting the immune response. CD95/CD95L interactions result in apoptosis of CD95-expressing cells mediated by crosslinking of CD95 by CD95L. Muschen and colleagues studied 40 breast malignancies for the expression of CD95L [65]. They observed that CD95L expression was positively correlated with the grade of malignancy with stage IV disease showing the highest rate of expression. In addition, in histologic analysis, they found that T cells within close proximity of tumor cells were predominantly apoptotic. The authors concluded that tumor cell-induced apoptosis of tumor-associated T cells is a potential mechanism of immunosuppression in breast cancer. To test this hypothesis, the investigators, in a separate study, examined apoptosis of Jurkat T cells following exposure to breast cancer cell lines [66]. They observed that the rate of apoptosis of Jurkat T cells was positively correlated with the levels of CD95L expressed on the tumor cell lines. Apoptotic ef-

fects were also enhanced by the addition of IFN γ suggesting a detrimental effect of this cytokine at the tumor site. Moreover, in cancer patients, depletion of CD4 $^{+}$ and CD8 $^{+}$ T cells in the peripheral blood was significantly correlated with CD95 expression suggesting both systemic and local immunosuppression.

Another mechanism of tumor-associated T cell dysfunction is acquired impairment in the signaling ability of the T cells. For example, in one study 9 of 14 breast cancer patients had impaired levels of one or more signaling molecules; zeta-chain, ZAP-70, p56lck, and MAP kinase phosphatase I [67]. This generalized immunosuppression is, at least partially, mediated by the accumulation of immature myeloid cells, Gr-1 $^{+}$ [68]. The TCR zeta-chain can be reduced in T cells by direct contact with Gr-1 $^{+}$ myeloid cells [69]. More recently, Gabrilovich and colleagues found that Gr-1 $^{+}$ cells purified from tumor-bearing mice directly inhibited tumor-antigen-specific CD8 T cells and had no impact on tumor-antigen-specific CD4 $^{+}$ T cells [70]. The effects of Gr-1 $^{+}$ cells depended on direct contact of the T cells with the Gr-1 $^{+}$ cells and could be abolished by inclusion of APC maturation factors (e.g. GM-CSF). These results suggest that therapies designed to promote the differentiation of myeloid cells, and hence, reverse zeta chain defects may improve the success of therapies targeting activation of tumor-specific T cells.

5. Immune-based strategies in minimal disease

Vaccine strategies for breast cancer are currently being designed to overcome tolerance and generate lasting immunity. As more effective immunization techniques are developed, the clinical application of breast cancer vaccines is being focused on patients with minimal disease. Vaccines targeting breast cancer are often administered in the presence of bulky disease and have had limited therapeutic success. Extrapolating from application of active immunization in infectious diseases, vaccines such as those targeting chicken pox or

influenza are given in the absence of infection and have only limited efficacy if administered after exposure to the pathogen. The goal of vaccination against breast cancer may be to elicit significant immunologic memory capable of rapidly expanding an antigen-specific T cell population in the presence of low levels of antigen encountered during cancer onset or early relapse. Indeed, vaccination against breast cancer will likely only be effective when disease is either absent or below the limit of detection. The two primary reasons for vaccinating in the absence of bulky disease are, (1) patients with existing disease are often immunosuppressed, and, (2) the response to repeated vaccination in the absence of disease is limited and reaches a plateau level that is insufficient to eradicate existing malignancy before death occurs.

However, vaccination in the presence of some tumor may be beneficial during generation of the immune response due to epitope spreading, apoptosis, and necrosis of tumor cells. Epitope spreading is an amplification of the immune response that results from extending immunity from one antigen to other secondary antigens also expressed by the tumor cells. Epitope or determinant spreading, is a phenomenon first described in autoimmune diseases including Theiler's murine encephalomyelitis virus-induced demyelinating disease, murine experimental autoimmune encephalitis, and diabetes in non-obese diabetic mice [71]. It is thought that initiating an immune response against a single antigen can elicit inflammation that leads to tissue damage. Tissue debris is taken up and other tissue antigens are cross-presented by APC to CD4⁺ T cells in the regional lymph nodes. These newly recruited antigen-specific CD4⁺ T cells could further exacerbate tissue destruction. In the setting of minimal residual tumor, inflammation at the tumor site induced by vaccination could potentially broaden the immune response as is seen in autoimmune disease. Although previous observations of epitope spreading have been confined to the CD4⁺ immune effector arm, recent studies

by Markiewicz and colleagues demonstrated that epitope spreading can occur following immunization with MHC class I-restricted peptides in the P815 tumor model. They observed that immunization against a single MHC class I-restricted peptide resulted in rejection of both P1A⁺ tumors and P1A⁻ tumors [72]. Analysis of the CTL response revealed that P1A⁺ immunization resulted in intermolecular epitope spreading of the immune response to another P815 tumor antigen, P1E. Thus, epitope spreading is a phenomenon applicable to both the CD4⁺ and CD8⁺ T cells. These results suggest that broadening the immune response with epitope spreading in the presence of tumor could potentially result in the generation of immunity that may minimize the emergence of antigen-loss variants [72].

Vaccination strategies to successfully immunize against breast cancer are varied. All techniques focus on enhancing the immunogenicity of the tumor cell or of a particular breast cancer-associated antigen. Vaccines that have been translated to the clinic include intact tumor cells transfected with co-stimulatory molecules to enhance immunogenicity, glycoprotein-based vaccines given directly after the administration of drugs such as cytoxan to alter the immune microenvironment, and protein-based vaccines administered with multiple adjuvants to create local inflammatory responses to augment the function of APC [73]. Methods of immunization that directly address the inadequacies of the pre-existent immune response to breast cancer are more likely to result in successful immunization. One area of vaccine development, focused on augmenting APC function, is the use of DC as vaccine adjuvants.

Much attention has been focused on the activation of DC during vaccination since DC initiate and sustain T cell-mediated immune responses. Target antigens can be delivered directly to ex vivo cultured DC followed by subsequent injection of the DC-based vaccine. DC can also be modulated in vivo by using adjuvants that target DC such as GM-CSF. Ex vivo generation of

antigen-pulsed DC has been used extensively in vaccination strategies and can result in the generation of substantial long-lived immunity [74]. For example, Brossart and colleagues conducted a small phase I/II trial to assess both the clinical and immunologic consequences of vaccinating breast and ovarian cancer patients with one of two vaccines consisting of ex vivo derived DC pulsed with either the HER-2/neu HLA-A2 peptides or MUC-1 HLA-A2 binding peptides [75]. Of 10 patients immunized, 5 developed peptide-specific responses as assessed by staining for intracellular IFN γ production by T cells. The peptide-specific T cells were also able to lyse HLA-matched HER-2/neu overexpressing tumor cell lines. Furthermore, in some of the responding patients, intermolecular epitope spreading occurred to CEA and MAGE-3, both of which are aberrantly expressed in some breast cancers, indicating potential inflammation at the tumor site as a result of vaccination. Vaccination resulted in partial regression in one patient and disease stabilization in another two patients. The disappointing clinical results are consistent with results of other trials and support the hypothesis that vaccines will likely be more efficacious in disease prevention rather than treatment of existing macroscopic disease.

Although the results of various DC immunization trials appear promising, the procedures used for isolating and loading DC are tedious and not yet broadly applicable to clinical practice [76]. The disadvantages of using DC loaded or pulsed with tumor-associated antigens include the uncertainty regarding the longevity of antigen presentation, the HLA restriction by the patient haplotype, and the relatively low number of known MHC class I, and in particular, MHC class II T helper related epitopes. Whole tumor cell preparations such as tumor lysates, apoptotic tumor cells, or DC-tumor cell fusions depend on the availability of tumor cells. In addition, the cytokines used in vitro to expand DC may affect their phagocytic activity and ability to migrate to DLN [77]. Given the problems with ex vivo generation of DC a

potential solution to the difficulties of DC culture would be to mobilize DC in vivo. Langerhans cells (LC) are skin DC and are the most effective APC present in the skin. The soluble factors present in the dermis that recruit and mature skin DC, LC are well known [78]. Similar to other APC, the LC role is to recognize, internalize, and process antigen encountered in the skin and to transport antigen to the DLN for T cell recognition. As LC move from the skin to the DLN they lose their ability to process antigen and express high levels of MHC and co-stimulatory molecules such as CD80 and CD86 [78]. The functional maturation of LC is affected by epidermal cytokines, in particular GM-CSF, IL-1 β , and TNF α and by stimulation with antigen-mediated, in part, by co-stimulatory molecules.

GM-CSF, administered intradermally, can act as a recruitment and maturation factor for LC [79,80]. GM-CSF, as an adjuvant can mobilize skin DC in vivo and has been used in vaccines targeting patients with breast cancer. Our laboratory has been conducting phase I clinical vaccine trials, using peptide-based vaccines, in breast and ovarian cancer patients with HER-2/neu-overexpressing tumors [74]. The HER-2/neu peptide-based vaccines consisted of HLA-class II binding peptides admixed with GM-CSF. Our initial clinical vaccination strategies have concentrated on eliciting a CD4 $^{+}$ T helper response; vigorous T helper response may serve to augment the production of HER-2/neu antibodies and/or HER-2/neu-specific CTL, both of which could be therapeutic. In addition, CD4 $^{+}$ T cells play a major role in the maintenance of immunologic memory. Patients were immunized once a month for 6 months and 38 patients completed the planned six vaccinations [81]. The majority of patients could be immunized to at least one of the peptides in their vaccine, indicative of immune competence of the selected population. In addition, over half of the patients developed HER-2/neu protein-specific immunity after peptide immunization. The HER-2/neu protein-specific T cell responses were similar in magni-

tude to responses generated to a foreign antigen, keyhole limpet hemocyanin. An interesting finding of this study was that greater than 75% of the patients who completed the course of vaccinations developed intramolecular epitope spreading to HER-2/neu peptides not contained within their vaccine preparation. Strikingly, epitope spreading was significantly associated with the development of HER-2/neu protein-specific immunity, consistent with the notion that epitope spreading is a function of natural endogenous processing of antigen [81]. In addition, immunity to HER-2/neu protein was durable and lasted for at least 1 year following the end of the vaccinations. An additional arm of the study evaluated immunization of patients with helper epitopes that encompassed within their natural sequence HLA-A2 binding motifs. The rationale for this approach is that CD4⁺ T cell help is required for the generation of CD8⁺ T cell responses that are long-lived. We examined immune responses to both the helper and HLA-A2 peptides in HLA-A2 patients selected to receive this vaccine formulation. The majority of patients developed both HER-2/neu peptide- and protein-specific T cell immunity. Peptide responses could be identified to both the longer class II epitopes as well as the HLA-A2 binding peptides. The peptide-specific CD8⁺ T cells were able to lyse HLA-matched, HER-2/neu expressing tumor cells and significant precursor frequencies persisted for greater than 1 year. These results demonstrate that durable T cell immunity can be generated in breast cancer patients and that vaccines can be developed that are capable of stimulating natural processing and presentation of antigen *in vivo*.

6. Immune-based strategies in advanced disease

The role of breast cancer vaccines may be in preventing cancer relapse or even, eventually, the development of tumors. However, the use of cancer vaccines as a treatment modality in eradicating established malignancy is questionable. Extrapo-

lating from infectious disease models, T cell precursor frequencies after influenza immunization may range from 1:25,000 to 1:5000. However, during an active infection, the antigen-specific T cell precursor frequency may achieve levels of 1:50 circulating T cells. Immune-based strategies in advanced stage breast cancer must focus on increasing the tumor-specific T cell precursor frequency to a therapeutic rather than protective level. Examples of therapeutic interventions that may potentially be effective in eradicating existing breast cancer include antigen-specific adoptive T cell therapy and the generation of a mixed chimera *in vivo* stimulating a graft vs. tumor effect, via allotransplantation.

The primary purpose of adoptive T cell therapy is to augment T cell responses over and above that achievable by vaccination alone. Clearly, vaccination can increase the number of immune T cells capable of recognizing and responding to antigen. Repeated vaccination further increases the number of immune effector cells, but eventually a plateau of responsiveness is reached and repeated immunizations do not appreciably change this value [82]. Adoptive immunotherapy may allow levels of immunity to be achieved which may mediate an antitumor response. Adoptive transfer of T cells have resulted in the infused cells representing as many as 1:2 of the host's lymphocytes [82]. Experiments in a murine model of breast cancer have shown that vaccination alone, in the neu-transgenic mouse, is effective only for prevention of disease and not treatment of established malignancy [73]. Mice, vaccinated with a neu-specific peptide-based vaccine, are able to resist a tumor challenge following the course of immunization. In contrast, if vaccination is started on the same day as a tumor implant is placed, tumors grow at the same rate in vaccinated as in control mice. Therefore, *in vivo* expansion of antigen-specific T cells must be limited or even suppressed. One potential method of increasing the number of antigen-specific T cells to the level needed to eliminate tumor is to expand T cells *ex vivo* followed by reinfusion. T cells derived

from neuTg mice immunized against neu were expanded *in vitro* with neu peptides [73]. The neu-specific lymphocytes were then infused into tumor-bearing mice resulting in tumor regression. Splenocytes from non-immunized animals were infused as a control and had no antitumor effect. Although adoptive immunotherapy has been successful in eradicating established disease in animal models of breast cancer [73] the approach has not been successfully translated to human clinical trials. Obstacles to the development of successful T cell therapy for human breast cancer have been the lack of (1) defined tumor antigens which would allow expansion of antigen-specific T cells, (2) a detailed understanding of the *in vitro* expansion requirements of T cells which would allow the generation of maximal numbers while retaining optimal antigen-specific function, and, (3) understanding the *in vivo* environment necessary for sustaining expansion *in vivo*. Over the last several years, several breast cancer tumor antigens such as HER-2/neu have been defined by virtue of a pre-existent immune response against that antigen in cancer patients [3,10,83]. In addition, the cytokine environment needed to expand a functional antitumor population and sustain it *in vivo* has been elucidated to some degree [82].

Recent studies have shown that it is technically feasible to readily expand breast antigen-specific T cells from patients who have been immunized with breast cancer vaccines [84]. The ease of *ex vivo* isolation and expansion may be related to a starting precursor frequency that has been boosted by vaccination [85]. As an example, a patient developed a significant increase in CD8⁺ precursor frequency to HER-2/neu HLA-A2 binding peptide, p369–377, after active immunization. Following vaccination, T cell clones specific for p369–377 were isolated by limiting dilution and characterized. A total of 21 p369–377 clones were generated from this patient. With the exception of two clones, all clones were CD3⁺. Eleven of the clones were CD8⁺/CD4[−]. Nine of the clones were CD4⁺/CD8[−], despite being

specific for an HLA-A2 binding peptide. The remaining five clones contained varying levels of both CD4 and CD8. The majority (19/21) of clones expressed the α/β TCR, but interestingly, two clones expressed the γ/δ TCR. Several of these clones could be induced to secrete IFN γ in response to p369–377 peptide stimulation. Several clones could lyse HLA-A2-transfected HER-2/neu-overexpressing tumor cells, including the γ/δ TCR expressing clones. A similar report by Reddish et al., demonstrates that breast cancer patients can generate MHC class I-restricted CTL against MUC-1-expressing adenocarcinomas following vaccination with a MUC-1 helper peptide [86]. Investigations, such as these, demonstrate *ex vivo* expansion and characterization of breast cancer-specific T cells is most likely facilitated by vaccination and may lay the foundation for the use of antigen-specific T cell infusions for the treatment of advanced stage breast cancer.

Another therapeutic intervention that may greatly increase the tumor-specific T cell precursor frequency is allogeneic hematopoietic stem cell transplantation (alloHSCT) [87]. AlloHSCT has been very effective in treating hematopoietic malignancy and a significant part of the curative potential of alloHSCT is due to reactivity of donor immune cells against host or tumor cell antigens referred to as the 'graft-versus-leukemia' or 'graft-versus-tumor' (GVT) effect [88–90]. The most compelling evidence for a cell-mediated GVT effect comes from recent observations that the infusion of allogeneic lymphocytes, or donor lymphocyte infusion (DLI), results in the remission of leukemic cells without any further cytotoxic therapy in patients who experienced relapse of disease after a traditional alloHSCT [91,92]. The effectiveness of DLI in inducing the remission of recurrent malignancy has been demonstrated for almost all hematologic malignancies although its efficacy varies across tumor subtypes [93,94].

Due to the success of allogeneic transplant in mediating an antitumor effect in hematopoi-

etic cancers, studies have been initiated investigating the approach in solid tumors such as breast cancer. Morecki and colleagues studied the effect of allogeneic adoptive T cell therapy on tumor growth in a murine transplant model using the 4T1 mammary carcinoma cell line, H-2^d [95]. Inoculation of 4T1 cells into syngeneic mice, BALB/c or (BALB/cXC57BL/6)F1, both of an H-2^d background, resulted in the development of lung tumors. Sub-lethally irradiated F1 mice were inoculated with 4T1 cells to simulate minimal residual disease. Mice then received immunocompetent splenocytes derived from naïve F1 mice, BALB/c mice that were syngeneic to the tumor but semi-allogeneic to the host, or from C57BL/6 mice that were allogeneic to the tumor and semi-allogeneic to the host. The survival of F1 tumor-bearing mice that were treated with allogeneic C57BL/6 splenocytes was significantly prolonged, as compared to mice given F1 or BALB/c-derived splenocytes syngeneic to 4T1 tumor cells. Furthermore, an efficient GVT reaction was demonstrated in vitro and in vivo with MHC-mismatched DBA/2 splenocytes from mice pre-sensitized by multiple injections of irradiated tumor or BALB/c-derived spleen cells [96].

Investigators at the National Cancer Institute used a murine metastatic breast cancer model to determine whether allo-specific donor CD8⁺ CTL of type 2 cytokine phenotype (Tc2 cells) mediate a GVT effect with reduced GVHD, as compared to allo-specific donor CD8⁺C TL of type 1 cytokine phenotype (Tc1 cells) [97]. A parent-into-F1 transplant model was established using B6, H-2^b, stem cell and T cell infusions into irradiated CB6F1 hosts, H-2^{b/d}. Mice were inoculated with an MMTV breast cancer line, TSA, H-2^d. Both Tc1 and Tc2 subsets lysed allogeneic targets, including the TSA breast cancer line. On average, transplanted mice receiving tumor, but no donor T cells, died of tumor at day 29 after transplant. Transplant recipients co-injected with tumor and Tc1 cells or Tc2 cells demonstrated a statistically significant prolongation of survival relative to controls. The GVT effect, mediated by

the Tc1 cells, was associated with histologic evidence of severe GVHD in all major target organs. In marked contrast, Tc2 recipients had greatly reduced histologic GVHD. These data indicate that immunocompetent cells allogeneic to the mammary carcinoma cells were able to inhibit tumor development in the primary host and to prevent tumor growth in the adoptive recipient, which suggests that allogeneic cell therapy may be an efficient antitumor therapy for breast cancer.

Although alloHSCT has shown promise as a therapeutic strategy in advanced stage renal cell carcinoma [98], whether the modality has relevant clinical implications in patients with breast cancer is still unknown but there is anecdotal clinical evidence to support this possibility [99–101]. Eibl and colleagues reported a 32-year-old woman with inflammatory breast cancer who received an allogeneic bone marrow transplant from an HLA-identical sibling [99]. The myeloablative conditioning regimen consisted of cyclophosphamide, thiotepe, and carboplatin. Resolution of the patient's liver metastases was observed simultaneously with the development of clinical GVHD in the first weeks after transplant. CTL, derived from the patient, were tested in a chromium-release assay against B and T lymphocytes of the patient and donor collected prior to transplant as well as against a panel of breast cancer cell lines. The T cells recognized host cells, but not HLA-identical donor cells. Recognition was MHC class I antigen-restricted. In addition, minor histocompatibility antigen (MiHA)-specific and MHC class I antigen-restricted cytotoxic T lymphocytes recognizing breast cancer cells were isolated from the peripheral blood of the patient. Four of eight breast cancer lines were recognized by CTL; three of them shared the HLA antigens HLA-A2, B44, and DR1. Similarly, Ueno and colleagues treated 10 metastatic breast cancer patients with high-dose chemotherapy (cyclophosphamide, BCNU, and thiotepe) and alloHSCT [100]. All patients engrafted and had hematologic recovery. Shortly after transplantation, one patient achieved a complete remission, five achieved a partial remis-

sion, and four had stable disease. In two patients metastatic liver lesions regressed with the onset of acute GHVD, suggesting a GVT effect.

These studies suggest a possible GVT effect in breast cancer; however, the results are not definitive and there is significant concern over subjecting patients to the potential risks associated with conventional allogeneic transplantation. These potential risks include GVHD and major organ dysfunction as a sequelae of myeloablative preparative regimens. The demonstration that an immune-mediated GVT effect plays a central role in the therapeutic efficacy of alloHSCT has led to the development of less intense conditioning regimens which are adequately immunosuppressive to permit the engraftment of donor hematopoietic stem cells. A variety of non-myeloablative conditioning regimens have been reported [102–107]. All regimens share the similar goal of providing sufficient immunosuppression to achieve allogeneic donor engraftment while attempting to minimize toxicity. Rizzieri and colleagues at Duke University have recently reported initial results using non-myeloablative alloHSCT in the setting of metastatic breast cancer [108]. Following a conditioning regimen of Campath 1H, fludarabine, and cyclophosphamide, five patients with metastatic breast cancer underwent alloHSCT. At a median follow-up of 90 days, three patients have been observed to have a partial response after transplantation. At the time of this report, four of the five patients have maintained a partial response or stable disease.

Several observations can be made from these early trials. The data indicate that engraftment of donor hematopoietic stem cells may occur with both fludarabine- and radiation-based regimens. However, as shown in other studies, there is great variability relative to the degree of donor engraftment, and graft rejection is a significant problem [102,109]. Although these regimens have provided many of the anticipated advantages, many of the problems that have been associated with myeloablative alloHSCT persist, e.g. GVHD, and will have to be addressed in future trials.

7. Conclusions

Breast cancer is an immunogenic tumor. Patients develop immune responses against specific proteins regulating the growth of their tumors but the immunity that occurs endogenously is not effective for tumor eradication. Both the immune microenvironment and the tumor microenvironment influence the initiation and propagation of breast cancer-specific immunity. As the complex interplay between T cells, cytokines, APC, tumor cells, and other immune effectors is unraveled, immunotherapy targeting breast cancer can be more rationally designed. Clinically, the application of immune-based treatment to breast cancer is dictated by stage of disease. Recent clinical studies are demonstrating the generation of immune responses after active immunization. Hopefully, advances defining immunity to breast cancer at the bench will result in similar successes in the application of the immunotherapies in the clinic.

8. Abbreviations

APC	antigen presenting cell
CTL	cytotoxic T cell
DC	dendritic cell
DLN	draining lymph node
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
MHC	major histocompatibility
MUC	mucin
NK	natural killer
NKT	natural killer T cell
neuTg	neu-transgenic
TCR	T cell receptor
TNF	tumor necrosis factor

Acknowledgements

This work is supported for M.L.D. by grants from the NIH, NCI (K24 CA85218 and R01 CA85374) as well as the Cancer Treatment and Research

Foundation. K.L.K. is supported by a DOD Breast Cancer Program Fellowship Award.

References

- Pardoll DM (1994) Tumour antigens. A new look for the 1990s. *Nature*, 369, 357.
- Houghton AN (1994) Cancer antigens: immune recognition of self and altered self. *J Exp Med*, 180, 1-4.
- Disis ML, Calenoff E, McLaughlin G et al. (1994) Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res*, 54, 16-20.
- Disis ML, Smith JW, Murphy AE, Chen W, Cheever MA (1994) In vitro generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu protooncogene protein. *Cancer Res*, 54, 1071-1076.
- Disis ML, Cheever MA (1996) Oncogenic proteins as tumor antigens. *Curr Opin Immunol*, 8, 637-642.
- Nanda NK, Sercarz EE (1995) Induction of anti-self-immunity to cure cancer. *Cell*, 82, 13-17.
- Bargmann CI, Hung MC, Weinberg RA (1986) The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature*, 319, 226-230.
- Coussens L, Yang-Feng TL, Liao YC et al. (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science*, 230, 1132-1139.
- Press MF, Cordon-Cardo C, Slamon DJ (1990) Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. *Oncogene*, 5, 953-962.
- Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA (1997) High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. *J Clin Oncol*, 15, 3363-3367.
- Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ (1995) Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *Proc Natl Acad Sci USA*, 92, 432-436.
- Vonderheide RH, Hahn WC, Schultze JL, Nadler LM (1999) The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity*, 10, 673-679.
- Reddish M, MacLean GD, Koganty RR et al. (1998) Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. *Int J Cancer*, 76, 817-823.
- Palmisano GL, Pistillo MP, Capanni P et al. (2000) Investigation of HLA class I downregulation in breast cancer by RT-PCR. *Hum Immunol*, 62, 133-139.
- Disis ML, Knutson KL, Schiffman K, Rinn K, McNeel DG (2000) Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. *Breast Cancer Res Treat*, 62(3), 245-252.
- Kalams SA, Walker BD (1998) The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J Exp Med*, 188, 2199-2204.
- Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA*, 89, 10578-10582.
- Reilly RT, Gottlieb MB, Ercolini AM et al. (2000) HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res*, 60, 3569-3576.
- Reilly RT, Machiels JP, Emens LA et al. (2000) The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res*, 61, 880-883.
- Surman DR, Dudley ME, Overwijk WW, Restifo NP (2000) Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. *J Immunol*, 164, 562-565.
- Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H (1998) The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med*, 188, 2357-2368.
- Disis ML, Grabstein KH, Sleath PR, Cheever MA (1999) Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin Cancer Res*, 5, 1289-1297.
- Zeng G, Touloukian CE, Wang X, Restifo NP, Rosenberg SA, Wang RF (2000) Identification of CD4+ T cell epitopes from NY-ESO-1 presented by HLA-DR molecules. *J Immunol*, 165, 1153-1159.
- Zeng G, Wang X, Robbins PF, Rosenberg SA, Wang RF (2000) CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. *Proc Natl Acad Sci USA*, 98, 3964-3969.
- Divino CM, Chen SH, Yang W, Thung S, Brower ST, Woo SL (2000) Anti-tumor immunity induced by interleukin-12 gene therapy in a metastatic model of breast cancer is mediated by natural killer cells. *Breast Cancer Res Treat*, 60, 129-134.
- Shi F, Ljunggren H, Sarvetnick N (2001) Innate immunity and autoimmunity: from self-protection to self-destruction. *Trends Immunol*, 22, 97-101.
- Nishimura T, Kitamura H, Iwakabe K et al. (2000) The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing

- dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol*, 12, 987–994.
28. Liu YY, Han TY, Giuliano AE, Cabot MC (1999) Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells. *J Biol Chem*, 274, 1140–1146.
 29. Carr A, Mullet A, Mazorra Z et al. (2000) A mouse IgG1 monoclonal antibody specific for N-glycolyl GM3 ganglioside recognized breast and melanoma tumors. *Hybridoma*, 19, 241–247.
 30. Dennis JW, Granovsky M, Warren CE (1999) Glycoprotein glycosylation and cancer progression. *Biochim Biophys Acta*, 1473, 21–34.
 31. Heath WR, Carbone FR (2001) Cross-presentation, dendritic cells, tolerance and immunity. *Annu Rev Immunol*, 19, 47–64.
 32. Wolfers J, Lozier A, Raposo G et al. (2001) Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med*, 7, 297–303.
 33. Bellone M (2000) Apoptosis, cross-presentation, and the fate of the antigen specific immune response. *Apoptosis*, 5, 307–314.
 34. Bell D, Chomarat P, Broyles D et al. (1999) In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J Exp Med*, 190, 1417–1426.
 35. Candido KA, Shimizu K, McLaughlin JC et al. (2001) Local administration of dendritic cells inhibits established breast tumor growth: implications for apoptosis-inducing agents. *Cancer Res*, 61, 228–236.
 36. Chen LK, Tourville B, Burns GF et al. (1986) Interferon: a cytotoxic T lymphocyte differentiation signal. *Eur J Immunol*, 16, 767–770.
 37. Djeu JY, Stocks N, Zoon K, Stanton GJ, Timonen T, Herberman RB (1982) Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes viruses. *J Exp Med*, 156, 1222–1234.
 38. Collart MA, Belin D, Vassalli JD, de Kossodo S, Vassalli P (1986) Gamma interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. *J Exp Med*, 164, 2113–2118.
 39. Buchmeier NA, Schreiber RD (1985) Requirement of endogenous interferon-gamma production for resolution of *Listeria monocytogenes* infection. *Proc Natl Acad Sci USA*, 82, 7404–7408.
 40. Nakajima C, Uekusa Y, Iwasaki M et al. (2001) A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. *Cancer Res*, 61, 3399–3405.
 41. Yao L, Pike SE, Setsuda J et al. (2000) Effective targeting of tumor vasculature by the angiogenesis inhibitors vasostatin and interleukin-12. *Blood*, 96, 1900–1905.
 42. Braun SE, Chen K, Foster RG et al. (2000) The CC chemokine CK beta-11/MIP-3 beta/ELC/Exodus 3 mediates tumor rejection of murine breast cancer cells through NK cells. *J Immunol*, 164, 4025–4031.
 43. Kim CH, Pelus LM, White JR, Applebaum E, Johanson K, Broxmeyer HE (1998) CK beta-11/macrophage inflammatory protein-3 beta/EBI1-ligand chemokine is an efficacious chemoattractant for T and B cells. *J Immunol*, 160, 2418–2424.
 44. Dieu MC, Vanbervliet B, Vicari A et al. (1998) Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med*, 188, 373–386.
 45. Kim CH, Pelus LM, Appelbaum E, Johanson K, Anzai N, Broxmeyer HE (1999) CCR7 ligands, SLC/6CKine/Exodus2/TCA4 and CKbeta-11/MIP-3beta/ELC, are chemoattractants for CD56(+)CD16(–) NK cells and late stage lymphoid progenitors. *Cell Immunol*, 193, 226–235.
 46. Esche C, Cai Q, Peron JM et al. (2000) Interleukin-12 and Flt3 ligand differentially promote dendropoiesis in vivo. *Eur J Immunol*, 30, 2565–2575.
 47. Josien R, Li HL, Ingulli E et al. (2000) TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med*, 191, 495–502.
 48. Fiebiger E, Meraner P, Weber E et al. (2001) Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. *J Exp Med*, 193, 881–892.
 49. Lynch DH, Andreasen A, Maraskovsky E, Whitmore J, Miller RE, Schuh JC (1997) Flt3 ligand induces tumor regression and antitumor immune responses in vivo. *Nat Med*, 3, 625–631.
 50. Brunda MJ, Luistro L, Warriar RR et al. (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med*, 178, 1223–1230.
 51. Lah TT, Hawley M, Rock KL, Goldberg AL (1995) Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages. *FEBS Lett*, 363, 85–89.
 52. Coulic PG, Ikeda H, Baurain JF, Chiari R (1999) Antitumor immunity at work in a melanoma patient. *Adv Cancer Res*, 76, 213–242.
 53. Sprent J, Kishimoto H (2001) The thymus and central tolerance. *Philos Trans R Soc Lond B Biol Sci*, 356, 609–616.

54. Lechler R, Chai JG, Marelli-Berg F, Lombardi G (2001) T-cell anergy and peripheral T-cell tolerance. *Philos Trans R Soc Lond B Biol Sci*, 356, 625–637.
55. Wolfram RM, Budinsky AC, Brodowicz T et al. (2000) Defective antigen presentation resulting from impaired expression of costimulatory molecules in breast cancer. *Int J Cancer*, 88, 239–244.
56. Meyer GC, Batrla R, Rudy W et al. (1999) Potential of CD80-transfected human breast carcinoma cells to induce peptide-specific T lymphocytes in an allogeneic human histocompatibility leukocyte antigens (HLA)-A2.1+-matched situation. *Cancer Gene Ther*, 6, 282–288.
57. Ohlen C, Kalos M, Hong DJ, Shur AC, Greenberg PD (2001) Expression of a tolerizing tumor antigen in peripheral tissue does not preclude recovery of high-affinity CD8+ T cells or CTL immunotherapy of tumors expressing the antigen. *J Immunol*, 166, 2863–2870.
58. Becker JC, Czerny C, Brocker EB (1994) Maintenance of clonal anergy by endogenously produced IL-10. *Int Immunol*, 6, 1605–1612.
59. Groux H, Bigler M, de Vries JE, Roncarolo MG (1996) Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med*, 184, 19–29.
60. Joss A, Akdis M, Faith A, Blaser K, Akdis CA (2000) IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur J Immunol*, 30, 1683–1690.
61. von Bernstorff W, Voss M, Freichel S et al. (2001) Systemic and local immunosuppression in pancreatic cancer patients. *Clin Cancer Res*, 7, 925s–932s.
62. Nemunaitis J, Fong T, Shabe P, Martineau D, Ando D (2001) Comparison of serum interleukin-10 (IL-10) levels between normal volunteers and patients with advanced melanoma. *Cancer Invest*, 19, 239–247.
63. Ochsenbein AF, Klenerman P, Karrer U et al. (1999) Immune surveillance against a solid tumor fails because of immunological ignorance. *Proc Natl Acad Sci USA*, 96, 2233–2238.
64. Dalyot-Herman N, Bathe OF, Malek TR (2000) Reversal of CD8+ T cell ignorance and induction of anti-tumor immunity by peptide-pulsed APC. *J Immunol*, 165, 6731–6737.
65. Muschen M, Moers C, Warskulat U et al. (1999) CD95 ligand expression in dedifferentiated breast cancer. *J Pathol*, 189, 378–386.
66. Uschen M, Moers C, Warskulat U, Even J, Niederacher D, Beckmann MW (2000) CD95 ligand expression as a mechanism of immune escape in breast cancer. *Immunology*, 99, 69–77.
67. Kurt RA, Urba WJ, Smith JW, Schoof DD (1998) Peripheral T lymphocytes from women with breast cancer exhibit abnormal protein expression of several signaling molecules. *Int J Cancer*, 78, 16–20.
68. Almand B, Clark JI, Nikitina E et al. (2001) Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol*, 166, 678–689.
69. Otsuji M, Kimura Y, Aoe T, Okamoto Y, Saito T (1996) Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses. *Proc Natl Acad Sci USA*, 93, 13119–13124.
70. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM (2001) Mechanism of immune dysfunction in cancer mediated by immature gr-1(+) myeloid cells. *J Immunol*, 166, 5398–5406.
71. Vanderlugt CJ, Miller SD (1996) Epitope spreading. *Curr Opin Immunol*, 8, 831–836.
72. Markiewicz MA, Fallarino F, Ashikari A, Gajewski TF (2001) Epitope spreading upon P815 tumor rejection triggered by vaccination with the single class I MHC-restricted peptide P1A. *Int Immunol*, 13, 625–632.
73. Knutson KL, Disis ML (2001) Expansion of HER-2/neu specific T cells ex vivo following immunization with a HER-2/neu peptide-based vaccine. *Clin Breast Cancer*, 2, 73–79.
74. Weber JS, Mule JJ (2001) How much help does a vaccine-induced T-cell response need? *J Clin Invest*, 107, 553–554.
75. Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W (2000) Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood*, 96, 3102–3108.
76. Fong L, Engleman EG (2000) Dendritic cells in cancer immunotherapy. *Annu Rev Immunol*, 18, 245–273.
77. Banchereau J, Briere F, Caux C et al. (2000) Immunobiology of dendritic cells. *Annu Rev Immunol*, 18, 767–811.
78. Kimber I, Cumberbatch M, Dearman RJ, Bhushan M, Griffiths CE (2000) Cytokines and chemokines in the initiation and regulation of epidermal Langerhans cell mobilization. *Br J Dermatol*, 142, 401–412.
79. Kaplan G, Walsh G, Guido LS et al. (1992) Novel responses of human skin to intradermal recombinant granulocyte/macrophage-colony-stimulating factor: Langerhans cell recruitment, keratinocyte growth, and enhanced wound healing. *J Exp Med*, 175, 1717–1728.
80. Disis ML, Bernhard H, Shiota FM et al. (1996) Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood*, 88, 202–210.
81. Disis ML, Gooley TA, Rinn K, Davis D, Piepkorn M, Cheever MA, Knutson KL, Schiffman K (2002) Intramolecular epitope spreading to the HER-2/neu

- protein occurs after active immunization with HER-2/neu peptide-based vaccine. (In preparation).
82. Cheever MA, Chen W (1997) Therapy with cultured T cells: principles revisited. *Immunol Rev*, 157, 177–194.
 83. Disis ML, Cheever MA (1998) HER-2/neu oncogenic protein: issues in vaccine development. *Crit Rev Immunol*, 18, 37–45.
 84. Knutson KL, Crosby P, Disis ML (2000) Isolation of HER-2/neu (HER2) specific T cell clones from a cancer patient following immunization with a HER2 peptide vaccine. *Proc Am Assoc Cancer Res*, 40, 310a.
 85. Knutson KL, Schiffman K, Disis ML (2001) Immunization with a HER-2/neu helper peptide vaccine generates of HER-2/neu CD8 T cell immunity in cancer patients. *J Clin Invest*, 107, 477–484.
 86. Reddish M, MacLean GD, Koganty RR et al. (1998) Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. *Int J Cancer*, 76, 817–823.
 87. Armitage JO (1994) Bone marrow transplantation. *N Engl J Med*, 330, 827–838.
 88. Buckner CD, Clift RA, Fefer A et al. (1974) Marrow transplantation for the treatment of acute leukemia using HL-A-identical siblings. *Transplant Proc*, 6, 365–366.
 89. Horowitz MM, Gale RP, Sondel PM et al. (1990) Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*, 75, 555–562.
 90. Maraninchi D, Gluckman E, Blaise D et al. (1987) Impact of T-cell depletion on outcome of allogeneic bone-marrow transplantation for standard-risk leukaemias. *Lancet*, 2, 175–178.
 91. Porter DL, Roth MS, McGarigle C, Ferrara JL, Antin JH (1994) Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia. *N Engl J Med*, 330, 100–106.
 92. Mackinnon S, Papadopoulos EB, Carabasi MH et al. (1995) Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood*, 86, 1261–1268.
 93. Kolb HJ, Schattenberg A, Goldman JM et al. (1995) Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients: European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. *Blood*, 86, 2041–2050.
 94. Collins RHJ, Shpilberg O, Drobyski WR et al. (1997) Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J Clin Oncol*, 15, 433–444.
 95. Morecki S, Yacovlev E, Diab A, Slavin S (1998) Allogeneic cell therapy for a murine mammary carcinoma. *Cancer Res*, 58, 3891–3895.
 96. Morecki S, Yacovlev E, Gelfand Y, Uzi I, Slavin S (2001) Cell therapy with preimmunized effector cells mismatched for minor histocompatible antigens in the treatment of a murine mammary carcinoma. *J Immunother*, 24, 114–121.
 97. Fuzak J, Jung U, Foley J, Eckhaus MA, Fowler DH (2000) Allospecific Tc2 cells mediate a graft-versus-tumor effect without ongoing GVHD in a murine model of metastatic breast cancer. *Blood*, 96, 551a.
 98. Childs R, Chernoff A, Contentin N et al. (2000) Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med*, 343, 750–758.
 99. Eibl B, Schwaighofer H, Nachbaur D et al. (1996) Evidence for a graft-versus-tumor effect in a patient treated with marrow ablative chemotherapy and allogeneic bone marrow transplantation for breast cancer. *Blood*, 88, 1501–1508.
 100. Ueno NT, Rondon G, Mirza NQ et al. (1998) Allogeneic peripheral-blood progenitor-cell transplantation for poor-risk patients with metastatic breast cancer. *J Clin Oncol*, 16, 986–993.
 101. Or R, Ackerstein A, Nagler A et al. (1998) Allogeneic cell-mediated immunotherapy for breast cancer after autologous stem cell transplantation: a clinical pilot study. *Cytokines Cell Mol Ther*, 4, 1–6.
 102. Giralt S, Estey E, Albitar M et al. (1997) Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood*, 89, 4531–4536.
 103. Slavin S, Nagler A, Naparstek E et al. (1998) Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood*, 91, 756–763.
 104. Khouri IF, Keating M, Korbling M et al. (1998) Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol*, 16, 2817–2824.
 105. Childs R, Clave E, Contentin N et al. (1999) Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: full donor T-cell chimerism precedes alloimmune responses. *Blood*, 94, 3234–3241.
 106. Spitzer TR, McAfee S, Sackstein R et al. (2000) Intentional induction of mixed chimerism and achievement of antitumor responses after nonmyeloablative conditioning therapy and HLA-matched donor bone marrow transplantation for refractory hematologic malignancies. *Biol Blood Marrow Transplant*, 6, 309–320.

107. Sandmaier BM, McSweeney P, Yu C, Storb R (2000) Nonmyeloablative transplants: preclinical and clinical results. *Semin Oncol*, 27, 78–81.
108. Rizzieri DA, Long GD, Vredenburgh JJ et al. (2000) Chimerism mediated immunotherapy using CAM-PATH T cell depleted peripheral blood progenitor cells (PBPC) with nonablative therapy provides reliable, durable allogeneic engraftment. *Blood*, 96, 521a.
109. McSweeney PA, Niederwieser D, Shizuru JA et al. (2001) Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood*, 97, 3390–3400.

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APPENDIX 6

Immunization with the intracellular domain (ICD) protein of HER-2/neu (HER2) generates predominantly Th2 immunity in neu-transgenic mice

Keith L. Knutson, Mike Ahlquist, and Mary L. Disis

Investigations from our group have shown that HER2 specific peptide-based vaccines elicit a Th1 T-cell response with only low-level antibody production. A HER2 specific antibody response, however, may be therapeutically effective. We questioned whether HER2 protein based vaccines; a strategy more typically associated with antibody formation would be more effective than peptides in eliciting HER2 antibody immunity. In this study we evaluated the T and B cell HER2 specific immune response after ICD protein vaccination in FVB mice transgenic for rat neu proto-oncogene (neu-Tg mice). Neu-Tg mice were immunized twice with 100 µg ICD protein with CFA as adjuvant 14 days apart. Fourteen days after the last vaccination, splenocytes were harvested for *in vitro* assessment of immunity. The median proliferative response (range) to ICD in sham vaccinated control animals, given as a stimulation index, was 1.2 (range 1.0-1.6). Animals immunized with ICD had a median stimulation index of 5.0 (range 2.8-6.3). ELISpot was used to assess the T cell response for specific Th phenotype. The median number of ICD-specific precursors in control animals, given as the number of precursors per 10^6 splenocytes, was 0 (no range). Four of five animals immunized with ICD demonstrated a median ICD-specific IL-4-secreting T cell precursor frequency of 282/ 10^6 splenocytes (range 176-1156). In contrast, only 1/5 mice demonstrated ICD-specific IFN-γ-secreting T cell precursors, with a frequency of 273. Control mice did not have detectable levels of ICD-specific antibody while ICD-immunized animals had a median peripheral blood anti-ICD antibody concentration of 2.1 mg/ml (range 0.9-3.9). These results show that ICD protein immunization can result in the development of neu-specific TH2 immunity with the generation of high antibody titers.

APPENDIX 7

An MHC Class II epitope derived from the neu oncoprotein will elicit differential levels of immunity after immunization in parental vs. mice transgenic for neu.

Keith L. Knutson, R. Mike Ahlquist, and Mary L. Disis

T helper cells are pivotal to the development of cell-mediated and humoral immunity. In this study we examined the immunogenic response to a putative MHC class II peptide, GVGSPYVSRLGICL, derived from the neu oncoprotein. The sequence of the peptide corresponds to amino acids 781-795 (p781) of rat neu. The immune response to p781 was evaluated in both neu-Tg and parental FVB mice following peptide immunization. Mice were immunized three times, 14 days apart with p781 with CFA/IFA or with CFA/IFA alone (control). While control mice did not demonstrate an immune response to p781, parental FVB mice developed a significant proliferative T cell response. In contrast, the neu-tg mice did not demonstrate immunity following vaccination suggesting increased tolerance to the epitope. When splenocytes from p781-immunized neu-Tg mice were cultured for 2 *in vitro* stimulations in the presence of peptide antigen and IL-2, peptide-specific T cells could be demonstrated, as assessed by proliferation response, indicating that p781-specific TCRs were retained in the neu-Tg mouse TCR repertoire. In addition to recovering a proliferation response to antigen *in vitro*, the cells secreted IFN- γ specifically in response to p781 but not an irrelevant MHC class II peptide. In this study we have identified an immunogenic MHC class II peptide, p781, present in the intracellular domain of the neu oncoprotein. Our immunization studies suggest that neu-Tg mice, in which this is a self peptide, may demonstrate some level of tolerance to this epitope. The identification of CD4 T cell helper epitopes of the neu oncoprotein may lead to improved strategies to modulate the immune response to prevent or treat neu-mediated tumors.

APPENDIX 8

Vaccination against the HER-2/neu oncogenic protein

H Bernhard¹, L Salazar², K Schiffman², A Smorlesi^{2,3}, B Schmidt¹,
K L Knutson² and M L Disis²

¹Technical University of Munich, Klinikum rechts der Isar, Department of Hematology and Oncology, Ismaningerstrasse 22, D-81664, Munich, Germany

²Division of Oncology, University of Washington, Seattle, Washington 98195–6527, USA

³Dipartimento Ricerche INRCA, via Birarelli, 8, 60100 Ancona, Italy

(Requests for offprints should be addressed to M L Disis, Box 356527, Oncology, University of Washington, Seattle, Washington 98195–6527, USA; Email: ndisis@u.washington.edu)

Abstract

The HER-2/neu oncogenic protein is a well-defined tumor antigen. HER-2/neu is a shared antigen among multiple tumor types. Patients with HER-2/neu protein-overexpressing breast, ovarian, non-small cell lung, colon, and prostate cancers have been shown to have a pre-existent immune response to HER-2/neu. No matter what the tumor type, endogenous immunity to HER-2/neu detected in cancer patients demonstrates two predominant characteristics. First, HER-2/neu-specific immune responses are found in only a minority of patients whose tumors overexpress HER-2/neu. Secondly, immunity, if detectable, is of low magnitude. These observations have led to the development of vaccine strategies designed to boost HER-2/neu immunity in a majority of patients. HER-2/neu is a non-mutated self-protein, therefore vaccines must be developed based on immunologic principles focused on circumventing tolerance, a primary mechanism of tumor immune escape. HER-2/neu-specific vaccines have been tested in human clinical trials. Early results demonstrate that significant levels of HER-2/neu immunity can be generated with active immunization. The T-cell immunity elicited is durable after vaccinations have ended. Furthermore, despite the generation of CD8⁺ and CD4⁺ T-cells responsive to HER-2/neu in a majority of patients, there is no evidence of autoimmunity directed against tissues that express basal levels of the protein. Cancer vaccines targeting the HER-2/neu oncogenic protein may be useful adjuvants to standard therapy and aid in the prevention of relapse in patients whose tumors overexpress the protein. Furthermore, boosting HER-2/neu-specific T-cell frequencies via active immunization may allow the *ex vivo* expansion of HER-2/neu-specific T-cells for use in adoptive immunotherapy, a therapeutic strategy directed against the treatment of established disease.

Endocrine-Related Cancer (2002) 9 33–44

Introduction

The HER-2/neu protein consists of a cysteine-rich extracellular ligand binding domain, a short transmembrane domain, and a cytoplasmic protein tyrosine kinase domain (Samanta *et al.* 1994, Olayioye *et al.* 2000). Binding of ligand to the extracellular domain (ECD) leads to dimerization that stimulates the intrinsic tyrosine kinase activity of the receptor and triggers autophosphorylation of specific tyrosine residues within the intracellular cytoplasmic domain (ICD). These phosphorylated residues then serve as anchoring sites for signaling molecules involved in the regulation of intracellular signaling cascades (Olayioye *et al.* 2000) and, thus, cell growth.

HER-2/neu is a self-protein expressed in a variety of tissues of epithelial origin and it plays a fundamental role in cellular proliferation and differentiation during fetal development. In adults, the HER-2/neu gene is present as a single copy in normal cells; however, amplification of the gene and resultant protein overexpression is seen in various cancers including breast, ovarian, colon, uterine, gastric, prostate, and adenocarcinoma of the lung. Furthermore, the overexpression of HER-2/neu is implicated in the malignant transformation of breast cancer (Allred *et al.* 1992, Stark *et al.* 2000, Allred *et al.* 2001) and is a biologically relevant protein in the pathogenesis of several other epithelial-based tumors, for example leading to the development of hormone resistance in prostate cancer (Craft *et al.* 1999).

Generating an active immune response directed against the HER-2/neu protein has several potential clinical advantages. Vaccination, if effective, would stimulate immunologic memory and could result in the prevention of relapse after standard therapy such as surgery and radiation had been administered. Relapse in patients with breast and ovarian cancer, in a high-risk category due to HER-2/neu protein overexpression, is a major clinical problem (Slamon et al. 1987). In addition, if antibody immunity could be generated by active immunization, durable levels of functional antibodies binding the ECD of the growth factor receptor could be elicited if appropriate epitopes were targeted. Compelling evidence that the HER-2/neu protein may be a reasonable vaccine candidate is the observation that patients with HER-2/neu overexpressing tumors have low level pre-existent immunity directed against the protein. Thus, over the past decade immunologic investigations focusing on the HER-2/neu protein have progressed from pre-clinical studies, defining HER-2/neu as a tumor antigen and using the pre-existent immune response to HER-2/neu present in cancer patients to develop vaccines, to clinical studies actively immunizing cancer patients against HER-2/neu and developing strategies to use HER-2/neu T-cell immunity as a treatment for established tumors.

The HER-2/neu oncogenic protein is a tumor antigen

Cancer patients have pre-existent immunity to HER-2/neu

Patients with a variety of cancers whose tumors overexpress HER-2/neu can have pre-existent antibody and T-cell immunity directed against the antigen. In general, immunity to HER-2/neu in cancer patients is of low magnitude and found only in a minority of patients with HER-2/neu-overexpressing tumors. Of note, cancer patients with pre-existing antibody or T-cell immunity to HER-2/neu show no evidence of autoimmune disease, suggesting that antibodies and antigen-specific T-cells that arise in association with overexpression of the oncogenic protein do not recognize cells expressing basal levels of HER-2/neu. Furthermore, immunity to HER-2/neu can be found in a variety of tumors, underscoring HER-2/neu as a shared tumor antigen in multiple different tissue types.

Antibody immunity directed against the HER-2/neu protein has been most widely studied. Investigations of HER-2/neu-specific antibodies in patients with breast cancer demonstrate that responses can be detected in patients with early stage disease, indicating that the presence of antibodies is not simply a reflection of tumor burden. HER-2/neu antibodies of titers $>1:1000$ were detected in 12 of 107 (11%) breast cancer patients compared with 0 of 200 (0%) controls (Disis et al. 1997). Detection of antibodies to HER-2/neu also

correlated with protein overexpression in the patient's primary tumor. A subsequent study evaluated 45 patients with advanced stage (III/IV) HER-2/neu-overexpressing breast and ovarian cancer for detection of pre-existent humoral immunity (Disis et al. 2000). Only 7% had detectable HER-2/neu-specific IgG antibodies. HER-2/neu protein overexpression is detected in 30–50% of colon cancers (Ward 1999). Antibodies to HER-2/neu have been found in the sera of patients with colon cancer; titers of $>1:1000$ were detected in 8 of 57 (14%) patients with colorectal cancer compared with 0 of 200 (0%) of the normal control population. Similar to the immune response in breast cancer patients, the ability to detect HER-2/neu antibodies correlated with overexpression of the protein in the patient's primary tumor (Ward 1999). Finally, HER-2/neu antibody immunity has been studied in prostate cancer. Detection of HER-2/neu-specific antibodies was significantly higher in patients with prostate cancer (15.5%, 31 of 200) compared with controls (2%, 2 of 100), and titers $\geq 1:100$ were most prevalent in the subgroup of patients with androgen-independent disease (McNeel et al. 2000).

Existent T-cell immunity to the oncogenic protein, both T-helper and cytotoxic T-cells (CTL), have been detected in patients with HER-2/neu overexpressing tumors. The identification of T-cells that can respond to HER-2/neu indicates that a portion of the T-cell repertoire will recognize this self-antigen. Furthermore, it may be more appropriate, when developing vaccine strategies designed to circumvent tolerance, to immunize patients to boost weak pre-existent responses rather than prime a *de novo* HER-2/neu-specific immune response in patients. Both CD4⁺ and CD8⁺ T-cell responses were evaluated in patients with advanced stage HER-2/neu-positive tumors (Disis et al. 2000). These patients had not received immunosuppressive chemotherapy for at least 30 days (median 5 months, range 1–75 months) prior to entry in the study. All patients were documented to be immunocompetent by delayed type hypersensitivity (DTH) testing using a skin anergy battery. Five of the 45 patients (11%) were found to have a detectable HER-2/neu protein-specific T-cell response as defined by a stimulation index >2.0 (range 2.0–7.9). A limited number of patients were human leukocyte antigen (HLA)-A2-positive and were evaluated for CD8⁺ T-cell immunity to a dominant HLA-A2 epitope derived from the HER-2/neu ECD, p369–377 (Fisk et al. 1995). None of the 8 patients evaluated had a precursor frequency $>1:100,000$ peripheral blood mononuclear cells (PBMC) to p369–377. However, 5 of 7 patients had significant levels of flu-specific immunity (mean 1:20 312, range 1:31 250–1:13 700) demonstrating anergy was not responsible for the lack of CD8⁺ response to the tumor antigen. Cytotoxic T-cells capable of lysing HER-2/neu-overexpressing tumor cell lines have been identified in both the peripheral blood and tumors of patients bearing a variety of HER-2/neu-overexpressing tumors. Early studies identified HER-2/neu-

specific CTL in the malignant ascites of HLA-A2-positive patients with HER-2/neu-overexpressing ovarian cancer (Ioannides *et al.* 1993). Similar investigations have isolated tumor-specific CTL from tumor-infiltrating lymphocytes of HLA-A2-positive HER-2/neu-overexpressing non-small-cell lung cancer (NSCLC). These CTLs specifically recognized HLA-2+HER-2/neu+ autologous and allogeneic NSCLC cell lines as well as HLA-matched and antigen-positive ovarian cancer cell lines (Yoshino *et al.* 1994). In addition, studies have identified HER-2/neu-specific CTL in patients with HER-2/neu-overexpressing breast, ovarian, renal cell, pancreatic, gastric, colon and lung cancers (Yoshino *et al.* 1994, Peoples *et al.* 1995, Brossart *et al.* 1998, Kono *et al.* 1998, Peiper *et al.* 1999). HER-2/neu-specific T-cells, isolated from cancer patients, can aid in the identification of epitopes appropriate for inclusion in vaccines.

HER-2/neu vaccine development focuses on strategies that will allow tolerance to be 'circumvented'

The development of peptide-based vaccines may be uniquely suited to stimulate immunity to a self-antigen such as HER-2/neu. The ability to mount an immune response is related to the immunodominance of specific antigenic determinants during natural immunologic processing of intact protein antigens. However, only a minor fraction of potential determinants in an antigen are presented in an immunodominant manner, while the remaining peptides are ignored (Sercarz *et al.* 1993). Usually, physiological mechanisms of immunologic tolerance to self prevent the induction of an immune response to self-proteins, such as HER-2/neu. Dominantly processed self-determinants are thought to be efficient in tolerance induction (Sercarz *et al.* 1993, Nanda & Sercarz 1995). However, in every self-antigen, there are sequestered determinants that are unable to induce tolerance and therefore could be immunogenic (Sercarz *et al.* 1993). These subdominant epitopes may trigger the threshold for T-cell activation and immune recognition if they are presented in abundance, such as when a self-protein becomes overexpressed. Overexpression of the HER-2/neu protein may result in subdominant peptides being presented in higher concentration in the major histocompatibility complex (MHC), thus triggering a T-cell response. Potentially, the processed peptide repertoire in MHC could be distinctly different in a tumor cell where a self-protein was overexpressed than in a non-malignant cell where a self-protein is present at basal levels. Abundance of subdominant epitopes in MHC molecules expressed on cancer cells could result in overexpressed self-proteins functioning as tumor-specific antigens. An alternative hypothesis is that subdominant epitopes are more effectively presented by highly activated and efficient antigen presenting cells (APC), such as dendritic cells (DC), or APC markedly activated by inflammatory signals

from the local immune microenvironment (Nanda & Sercarz 1995).

Computer modeling programs have been effective in predicting potential immunogenic epitopes of self-proteins such as HER-2/neu, and early studies have focused on evaluating constructed peptides for signs of immune reactivity in patients with HER-2/neu-positive tumors (Disis & Cheever 1998). MHC class I-binding epitopes can be identified and corresponding synthetic peptides tested for their capacity to induce peptide- and tumor-specific CTL derived from healthy individuals or cancer patients (Rongcun *et al.* 1999). Using this method, Rongcun and colleagues identified four HER-2/neu-specific HLA-A2.1 restricted CTL epitopes: HER2(9₃₆₉), HER2(9₄₃₅), HER2(9₆₈₉), and HER2(9₆₆₅) which were able to elicit CTL that specifically killed peptide-sensitized target cells, and most, importantly, a HER-2/neu-transfected cell line and autologous tumor cells. In addition, CTL clones specific for HER2(9₃₆₉), HER2(9₄₃₅), and HER2(9₆₈₉) epitopes were isolated from tumor-specific CTL lines, further demonstrating the immunogenicity of these epitopes. A similar strategy involves defining candidate epitopes by their MHC-binding motif and class I affinity (Keogh *et al.* 2001). Identified high affinity peptides are then tested for *in vitro* reactivity with PBMC from normal donors and the ability to induce tumor-reactive CTLs. A potential problem in the development of CTL epitope-based vaccines is the large degree of MHC polymorphism. However, it is now known that HLA class I molecules can be divided into several families or supertypes based on similar peptide-binding repertoires (Keogh *et al.* 2001). For example, the A2 supertype consists of at least eight related molecules, and of these the most frequently observed are HLA-A*0201, A*0202, A*0203, A*0206, and A*6802. In addition, the A2 supertype is expressed in all major ethnicities – in the 39–46% range of most common populations. Many peptides that bind A*0201 also exhibit degenerate binding (binding to multiple alleles); thus an A2 supertype multi-epitope vaccine could be designed to provide broad population coverage (Keogh *et al.* 2001).

The relationship between class I affinity and tumor antigen epitope immunogenicity is of importance because tissue-specific and developmental tumor antigens, such as HER-2/neu, are expressed on normal tissues at some point in time at some location within the body. T-cells specific for these self-antigens could be functionally inactivated by T-cell tolerance; however, several studies have now shown CTL responses to tumor epitopes in both normal donors and cancer patients, indicating that tolerance to these tumor antigens, if it exists at all, is incomplete (Kawashima *et al.* 1998, Keogh *et al.* 2001). Whether or not T-cells recognizing high-affinity epitopes have been selectively eliminated, leaving a repertoire capable of recognizing only low-affinity epitopes, is not known. Further studies evaluated several peptides derived from four different tumor antigens, p53, HER-2/neu,

carcinoembryonic antigen (CEA), and MAGE proteins, for their capacity to induce CTL *in vitro* capable of recognizing tumor target lines (Keogh et al. 2001). In order to increase the likelihood of overcoming tolerance, fixed anchor analogs that demonstrate improved HLA-A*0201 affinity and binding were used. Forty-two wild-type and analog peptides were screened. All the peptides bound HLA-A*0201 and two or more additional A2 supertypes alleles with an IC_{50} of 500 nM or less. A total of 20/22 wild-type and 9/12 single amino acid substitution analogs were found to be immunogenic in primary *in vitro* CTL induction assays, using normal PBMCs and monocyte-derived dendritic cells as APC. Cytotoxic T-cells generated by 13/20 of the wild-type epitopes and 6/9 of the single substitution analogs tested recognized HLA-matched antigen-bearing cancer cell lines. Further analysis revealed that recognition of naturally processed antigen was correlated with high HLA-A2.1 binding affinity (IC_{50} = 200 nM or less; $P \geq 0.008$), suggesting that high binding affinity epitopes are frequently generated and can be recognized as a result of natural antigen processing. Studies such as these demonstrate that recognition of self-tumor antigens is within the realm of the T-cell repertoire and that binding affinity may be an important criterion for epitope selection. Peptide-based vaccines have been found to be a strategy that will allow tolerance to be circumvented in animal models of new immunization (Disis et al. 1996b). Therefore, rapid prediction and screening of HER-2/neu-specific peptide epitopes may aid the development of clinical vaccines for use in the treatment of HER-2/neu overexpressing tumors.

Another aspect of peptide epitope prediction would be to identify peptide portions of the HER-2/neu ECD that would be appropriate to target with an antibody response. Several monoclonal antibodies against the HER-2/neu ECD have been isolated and one such antibody, trastuzumab, has demonstrated clinical efficacy in the treatment of metastatic breast cancer (Vogel et al. 2001). Although many HER-2/neu-specific antibodies inhibit the growth of cancer cells, some antibodies have no effect on cell growth while others even actively stimulate cancer growth (Yip et al. 2001). This wide range of biological effects is thought to be related to the epitope specificity of the antibodies and to consequent changes in receptor signaling (Yip et al. 2001). An alternative to the use of passive antibody therapy would be active immunization against the HER-2/neu ECD. However, inappropriately induced immune responses could have untoward effects on cancer growth. Therefore, it is crucial to identify epitopes on HER-2/neu that are targeted by stimulatory and inhibitory antibodies in order to ensure the induction of a beneficial endogenous antibody response.

In a recent study, investigators constructed HER-2/neu gene fragment phage display libraries to epitope-map a number of HER-2/neu-specific antibodies with different biological effects on tumor cell growth (Yip et al. 2001). Regions

responsible for opposing effects of antibodies were identified and then used to immunize mice. The epitopes of three antibodies, N12, N28, and L87 were successfully located to peptide epitope binding regions of HER-2/neu. While N12 inhibited tumor cell proliferation, N28 stimulated the proliferation of a subset of breast cancer cell lines overexpressing HER-2/neu. The peptide region recognized by N12 was used as an immunogen to selectively induce an inhibitory immune response in mice. Mice immunized with the peptide developed antibodies that recognized both the peptide and native HER-2/neu. More importantly, HER-2/neu-specific antibodies purified from mouse sera were able to inhibit up to 85% of tumor cell proliferation *in vitro*. This study provides direct evidence of the function-epitope relationship of HER-2/neu-specific antibodies generated by active immunization. Using peptide regions that contain multiple inhibitory B cell epitopes is likely to be superior to the use of single epitope immunogens (Dakappagari et al. 2000). Current clinical trials of HER-2/neu vaccines largely focus on the use of peptide epitopes as immunizing antigens.

Human clinical trials of vaccines targeting the HER-2/neu oncogenic protein

Stimulating a cytotoxic T-cell response to HER-2/neu *in vivo*

The cytotoxic T-cell has been considered the primary effector cell of the immune system capable of eliciting an anti-tumor response. The predominant experimental method of stimulating a CTL response *in vivo* has been to vaccinate individuals with tumor cells or viruses recombinant for tumor antigens that can infect viable cells, so that proteins are exposed inside the cell and are processed and presented in the class I MHC antigen processing pathway. An alternative effective vaccination strategy to elicit CTL uses a soluble peptide that is identical or similar to naturally processed peptides that are present in class I MHC molecules along with adjuvant. An HLA-A2 binding peptide, p369–377, derived from the protein sequence of HER-2/neu ECD has been used extensively in clinical trials to generate CTL specific for cells overexpressing HER-2/neu *in vivo* via active immunization.

In an initial clinical study, HLA-A2-positive patients with metastatic HER-2/neu-overexpressing breast, ovarian, or colorectal carcinomas were immunized with 1 mg p369–377 admixed in incomplete Freund's adjuvant (IFA) every 3 weeks (Zaks & Rosenberg 1998). Peptide-specific CTL were isolated and expanded from the peripheral blood of patients after 2 or 4 immunizations. The CTL could lyse HLA-matched, peptide-pulsed, target cells but could not lyse HLA-matched tumors expressing the HER-2/neu protein. Even

when tumors were treated with interferon γ (IFN γ) to upregulate class I, the CTL lines generated from the patients would not respond to the peptide presented endogenously on tumor cells. An additional problem in using single HLA binding epitopes is that, without CD4⁺ T-cell help, responses generated are short lived and non-durable. More recently, a similar study was performed, immunizing patients with p369–377 using granulocyte macrophage colony-stimulating factor (GM-CSF) as an adjuvant (Knutson *et al.* 2002). GM-CSF is a recruitment and maturation factor for skin DC, Langerhans cells (LC) and, theoretically, may allow more efficient presentation of peptide epitopes than standard adjuvants such as IFA. Six HLA-A2 patients with HER-2/neu-overexpressing cancers received 6 monthly vaccinations with 500 μ g HER-2/neu peptide p369–377, admixed with 100 μ g GM-CSF. The patients had either stage III or stage IV breast or ovarian cancer. Immune responses to the p369–377 were examined using an IFN γ ELISPOT assay. Prior to vaccination, the median precursor frequency, defined as precursors/10⁶ PBMC, to p369–377 was not detectable. Following vaccination, HER-2/neu peptide-specific precursors developed to p369–377 in just 2 of 4 evaluable subjects. The responses were short-lived and not detectable at 5 months after the final vaccination. Immunocompetence was evident as patients had detectable T-cell responses to tetanus toxoid and influenza. These results demonstrate that HER-2/neu MHC class I epitopes can induce HER-2/neu peptide-specific IFN γ -producing CD8⁺ T-cells. However, the magnitude of the responses was low as well as short-lived. Theoretically, the addition of CD4⁺ T-cell helper epitopes would allow the generation of lasting immunity.

A successful vaccine strategy in generating peptide-specific CTL capable of lysing tumor expressing HER-2/neu and resulting in durable immunity involved immunizing patients with putative T-helper epitopes of HER-2/neu which had, embedded in the natural sequence, HLA-A2 binding motifs of HER-2/neu. Thus, both CD4⁺ T-cell helper epitopes and CD8⁺ specific epitopes were encompassed in the same vaccine. In this trial, 19 HLA-A2 patients with HER-2/neu-overexpressing cancers received a vaccine preparation consisting of putative HER-2/neu helper peptides (Knutson *et al.* 2001). Contained within these sequences were the HLA-A2 binding motifs. Patients developed both HER-2/neu-specific CD4⁺ and CD8⁺ T-cell responses. The level of HER-2/neu immunity was similar to viral and tetanus immunity. In addition, the peptide-specific T-cells were able to lyse tumor. The responses were long-lived and detectable for greater than 1 year after the final vaccination in selected patients. These results demonstrate that HER-2/neu MHC class II epitopes containing encompassed MHC class I epitopes are able to induce long-lasting HER-2/neu-specific IFN γ -producing CD8⁺ T-cells.

Stimulating a T helper cell response to HER-2/neu *in vivo*

Pre-existent immune responses to HER-2/neu are of low magnitude. Therefore, before an assessment as to the anti-tumor effect of HER-2/neu-specific immunity can be made, the level of immunity should be augmented. Stimulating an effective T helper response is a way to boost antigen-specific immunity as CD4⁺ T-cells generate the specific cytokine environment required to support an evolving immune response. Furthermore, either CTL or an antibody immunity may have an effect on HER-2/neu-overexpressing tumor growth. Targeting CD4⁺ T-cells in a vaccine strategy would result in the potential to augment either of these arms of the immune system.

Putative T helper subdominant peptide epitopes, derived from the HER-2/neu protein sequence, were predicted by computer modeling and screened for immune reactivity using PBMC from patients with breast and ovarian cancer (Disis & Cheever 1998). Vaccines were generated each composed of three different 15–18 amino acid long HER-2/neu peptides. Patients with advanced stage HER-2/neu-overexpressing breast, ovarian, and non-small-cell lung cancer were enrolled and 38 patients finished the planned course of 6 immunizations (Disis *et al.* 2002a). Patients received 500 μ g of each peptide admixed in GM-CSF in an effort to mobilize LC *in vivo* as an adjuvant to peptide immunization (Disis *et al.* 1996a). Ninety-two percent of patients developed T-cell immunity to HER-2/neu peptides and over 60% to a HER-2/neu protein domain. Thus, immunization with peptides resulted in the generation of T-cells that could respond to protein processed by APC. Furthermore, at 1 year follow-up, immunity to the HER-2/neu protein persisted in 38% of patients. Immunity elicited by active immunization with CD4⁺ T helper epitopes was durable.

An additional finding of this study was that epitope spreading was observed in 84% of patients and significantly correlated with the generation of HER-2/neu protein-specific T-cell immunity ($P = 0.03$). Epitope, or determinant spreading, is a phenomenon first described in autoimmune disease (Lehmann *et al.* 1992) and has been associated with both MHC class I- and MHC class II-restricted responses (Vanderlugt & Miller 1996, el-Shami *et al.* 1999). Epitope spreading represents the generation of an immune response to a particular portion of an immunogenic protein and then the natural spread of that immunity to other areas of the protein or even to other antigens present in the environment. In this study, epitope spreading reflected the extension of a significant T-cell immune response to portions of the HER-2/neu protein that were not contained in the patient's vaccine. How does epitope spreading develop? Theoretically, a broadening of the immune response may represent endogenous processing of antigen at sites of inflammation initiated by a

specific T-cell response or 'driver clone' (Sercarz 2000). That is, the initial immune response can create a microenvironment at the site of the tumor that enhances endogenous immune effector cells present locally. These immune cells, e.g. APC and T-cells, may begin to respond more effectively to tumor antigen that is present in the body. Another recently reported vaccine trial immunizing breast and ovarian cancer patients with autologous DC pulsed with mucin-1 or HER-2/neu peptides resulted in epitope spreading (Brossart et al. 2000). In this trial, 10 patients were immunized and half the patients developed CD8⁺ T-cell precursors to their immunizing peptides. Moreover, some patients developed new immunity to other tumor antigens expressed in their cancers, such as CEA and MAGE-3.

Most clinical trials of cancer vaccines focus on the detection of a newly generated immune response or the magnitude of the antigen-specific immune response elicited after active immunization. However, the detection of epitope spreading indicating an immune microenvironment capable of producing an endogenous polyclonal immune response may be an endpoint that could potentially reflect an improved clinical outcome. Recent studies have evaluated vaccine strategies focused to maximize the role of the most efficient APC, the DC or skin LC, in eliciting effective immunity to self. One such strategy is to use cytokines involved in DC production and maturation as vaccine adjuvants. Flt3-ligand (FL) is a cytokine which, when administered systemically, can increase numbers of circulating DC greater than 40-fold (Maraskovsky et al. 2000). Human DC generated by the administration of FL have been shown to be functional and can stimulate T-cells *in vitro* (Maraskovsky et al. 2000). Furthermore, activation of DC *in vivo* by FL has been shown to be an effective way of circumventing tolerance during active immunization in animal models (Pulendran et al. 1998). Studies have been performed in the neu transgenic mouse, immunizing the animals to a self-tumor antigen, neu, using FL as a vaccine adjuvant to mobilize DC *in vivo* (Smorlesi et al. 1999). The timing of vaccine administration corresponded to the kinetics of *in vivo* DC mobilization in animals (Maraskovsky et al. 1996, Lynch 1998): early administration when few circulating DC are present, midpoint administration when DC precursors are increasing in the peripheral blood, and finally vaccination at the end of the FL cycle when DC are at peak concentrations. Thus, during a 10-day administration of FL a HER-2/neu ICD protein vaccine was administered at 3 time-points. Animals receiving the vaccine at midpoint in the FL cycle generated HER-2/neu ICD-specific immunity whereas mice immunized at the end of the FL cycle did not. In general, neu-specific immunity generated using FL resulted in T-cells that predominantly secreted IFN γ , a Type 1 associated cytokine, rather than interleukin (IL)-4, a Type 2 associated cytokine (Smorlesi et al. 1999).

On the basis of these data generated in rodent models, 10 patients with HER-2/neu-overexpressing cancers were

enrolled to receive a HER-2/neu peptide-based vaccine targeting the ICD of the HER-2/neu protein (Disis et al. 2002b). The peptides in the vaccine were the same as those used in one of the arms of the trial described above (Disis et al. 2002a) admixed with GM-CSF alone as an adjuvant. All patients received FL 20 μ g/kg per day s.c. for 14 days. Five patients received the HER-2/neu peptide-based vaccine alone intradermally at midpoint in one FL cycle and 5 patients received the vaccine admixed with 150 μ g GM-CSF intradermally at midpoint in the FL cycle. T-cell proliferative responses to HER-2/neu peptides and ICD protein were not significantly boosted in either FL arm. However, including FL as a vaccine adjuvant was effective in boosting the precursor frequency of IFN γ -secreting HER-2/neu-specific T-cells. After the completion of all immunizations, all 4 patients in each group developed detectable IFN γ -producing T-cells specific for the ICD protein: FL alone arm, mean frequency 1:5000 (range 1:30 000–1:2000) and FL and GM-CSF arm, mean frequency 1:2500 (range 1:5700–1:1500). The small sample size of each group, however, did not allow a statistically significant comparison of immune responses between the FL alone and FL with GM-CSF arms.

Recent investigations have demonstrated that FL and GM-CSF may stimulate different subsets of DC *in vivo* and that the cytokine microenvironment elicited, either Type 1 or Type 2, is markedly influenced by the particular DC subset generated. Evaluating a murine model of cancer using tumors engineered to express either GM-CSF or FL, demonstrated that GM-CSF engineered cells were more potent in inducing an anti-tumor response (Mach et al. 2000). GM-CSF elicited a diverse cytokine environment consisting of both T helper cell (Th) 1 and Th2 immune effectors. In contrast, immune responses generated with FL-expressing tumor cells were specifically restricted to a Th1 phenotypic response (Mach et al. 2000). Data from human clinical trials using FL as a vaccine adjuvant support the notion that FL is associated with the development of a strong Type 1 response. The detection of antigen-specific cytokine production without concomitant measurable clonal proliferation has been reported and is potentially a reflection of a strongly restricted Type 1 response (van der Veen et al. 1999). However, the increased number of APCs stimulated through the use of FL did function to present antigen and was verified by an additional finding. The addition of FL in the vaccine regimen was associated with the development of autoimmune phenomena in some patients. In general, the vaccine regimens including FL were well tolerated. One patient had grade 1 serologic abnormalities (anti-nuclear antibody (ANA), anti single strand antibody (anti-SSA), anti double strand DNA (anti-dsDNA)). The second patient, who had stage IV breast cancer, developed grade 2 toxicity with serologic abnormalities and self limiting Sicca syndrome characterized by dry eyes and dry mouth 3 months after the completion of the vaccine regimen. This patient did not develop any detectable immunity to HER-2/

neu peptides or protein after active immunization. None of the patients immunized on any reported HER-2/neu-specific vaccine trial developed any evidence of autoimmune phenomenon directed against tissues that express basal levels of HER-2/neu.

Active immunization results in increased precursor frequency and diversity of the T-cell repertoire

The generation of HER-2/neu antigen-specific T-cells from unprimed individuals is difficult. Isolation and expansion of T-cells from the peritoneal fluid of patients with HER-2/neu-overexpressing ovarian cancer require multiple *in vitro* stimulations (IVS) (Peoples *et al.* 1995, Fisk *et al.* 1996). In addition, deriving HER-2/neu peptide-specific T-cells from unprimed donors entails laborious and lengthy expansion techniques (Disis *et al.* 1994). By boosting HER-2/neu precursor frequency after peptide immunization, one can more readily expand and clone HER-2/neu-specific T-cells from the peripheral blood of patients with HER-2/neu-overexpressing breast cancers as compared with naive donors (Knutson & Disis 2001). The evaluation of a significant number of antigen-specific T-cells after active immunization (Peoples *et al.* 1995) will begin to allow us to dissect the tumor antigen-specific immune response for clues concerning the nature of the tumor-specific T-cells, affinity to MHC receptor, and potential for therapeutic efficacy.

A recent investigation demonstrated the marked phenotypic diversity of the vaccinated response (K L Knutson & M L Disis, unpublished observations). T-cell clones specific for HER-2/neu HLA-A2 peptide p369–377 were isolated from an ovarian cancer patient who had been vaccinated with HER-2/neu helper epitopes that contained HLA-A2-binding CTL epitopes within their sequences. Throughout the course of immunization, PBMC from this patient showed strong proliferative responses to the HER-2/neu helper epitope, p369–384. Following vaccination, T-cell clones specific for p369–377, the HLA-A2 binding peptide, were isolated by limiting dilution and then characterized. The responding T-cell repertoire generated was both phenotypically and functionally diverse. A total of 21 p369–377 T-cell clones were isolated from this patient. Sixteen of the clones were CD8⁺ and 5 of the clones were CD4⁺, despite being generated with an HLA-A2-binding peptide. The CD4 molecule is known to play a critical role in stabilizing the interaction of HLA class II peptide with the T-cell receptor (TCR) and its presence promotes the expansion of low-affinity peptide-specific TCRs and ensures a diverse T-cell response. Although CD4⁺ T-cells are predominantly associated with responding to peptides associated with HLA class II, at lower frequencies CD4 plays a role in regulating HLA class I-restricted T-cells, particularly T-cells associated with cancers such as melanoma, colon, and pancreatic cancer (de Vries & Spits 1984,

Somasundaram *et al.* 2000). Nineteen of 21 clones expressed the $\alpha\beta$ TCR. The remaining 2 clones expressed the $\gamma\delta$ TCR. Clones could lyse HLA-A2-transfected HER-2/neu-overexpressing tumor cells as well as peptide-loaded HLA matched cells. In addition to their lytic capabilities, these clones could be induced to produce IFN γ specifically in response to p369–377 peptide stimulation. The $\gamma\delta$ TCR clones expressed CD8 and lysed HLA-A2 HER-2/neu-positive tumor cells, but not HLA-A2-negative HER-2/neu-overexpressing tumor cells. $\gamma\delta$ T-cells are involved in a wide range of immune responses to infectious and non-infectious diseases, including malaria, mycobacterial infections, cancers, as well as autoimmune disorders such as multiple sclerosis (Boismenu & Havran 1998). Often, $\gamma\delta$ T-cells clones are isolated from the tumor-infiltrating lymphocyte population of many tumors, including dysgerminoma (Zhao *et al.* 1995), seminoma (Zhao *et al.* 1995), renal carcinoma (Choudhary *et al.* 1995), lung (Yu *et al.* 1999), colorectal (Watanabe *et al.* 1995), and melanoma (Bachelez *et al.* 1992). The recruitment to and role of these unique cells in mediating antitumor immunity is unknown. In autoimmune diseases, some pathological observations have been attributed to infiltrating $\gamma\delta$ TCR T-cells. It is unknown if the autoreactive $\gamma\delta$ TCR T-cells respond secondarily to damaged and stressed tissue (Hayday & Geng 1997) or if they initiate autoimmunity directly. One hypothesis is that, given their broad range of regulation by multiple mechanisms of antigen presentation and natural localization to epithelial tissue, $\gamma\delta$ TCR T-cells are sentinels for the immune system and are capable of alerting the immune system to the presence of danger (e.g. infection, tumors, etc.). These results suggest that a tumor antigen-specific T-cell response can be markedly polyclonal at multiple levels, including T-cell subset and TCR. Perhaps functional immunity directed against specific self-antigens mimics the pathogenic pathways of autoimmune disease more closely than anticipated.

Defining the clinical role of cancer vaccines

The cumulative data from the limited number of completed phase I clinical trials using HER-2/neu peptide-based vaccines to immunize against the HER-2/neu protein indicate that patients can be vaccinated against this self-tumor antigen. Vaccination offers a potential therapeutic strategy to prevent the relapse of disease by establishing an effective memory response targeting HER-2/neu. In addition, active immunization can provide a polyclonal T-cell population specific for the tumor antigen that can be expanded and used in adoptive immunotherapy. Extrapolating from the experience with infectious disease vaccines, active immunization has the greatest chance of therapeutic efficacy if used in a minimal disease state, not against a rapidly growing drug-resistant tumor. Preclinical investigations have demonstrated that eradicating established tumors in cancer patients will

require the generation of high levels of tumor-specific immunity, levels which cannot be achieved by vaccination but rather by infusion of competent T-cells, i.e. adoptive T-cell therapy (Cheever & Chen 1997). Extrapolating from infectious disease models, T-cell precursor frequencies after influenza immunization may range from 1:25 000–1:5000. However, during an active infection, the antigen-specific T-cell precursor frequency may achieve levels of 1:50 circulating T-cells. Clearly, vaccination can increase the number of immune T-cells capable of recognizing and responding to antigen. Repeated vaccination further increases the number of immune effector cells, but eventually a plateau of responsiveness is reached and repeated immunizations do not appreciably change this value. Adoptive transfer of T-cells has resulted in the infused cells representing 1:2 of the host's lymphocytes (Cheever & Chen 1997). Adoptive immunotherapy may allow levels of immunity to be achieved that may be able to treat bulky disease.

HER-2/neu-specific T lymphocytes for adoptive T-cell therapy

Patients with established, rapidly growing tumors can have an impaired cellular and humoral immune system. Therefore, it might be difficult to activate immunological defense mechanisms by vaccination. The rationale of adoptive T-cell therapy is based on the attempt to circumvent this tolerizing tumor microenvironment by taking out the anergic, potentially tumor-reactive T-cells from the cancer-bearing patient and subsequently activating these T-cells *ex vivo*. Following expansion of tumor-reactive T-cells *in vitro*, great numbers of T-cells can be adoptively transferred to the immunosuppressed patient. In this way, a high frequency level of *ex vivo* activated tumor-reactive T-cells can be achieved *in vivo*. This level of immunity cannot be achieved by active immunization.

The therapeutic efficacy of adoptively transferred T-cells was first documented in bone marrow recipients at risk for virus infections or virus-induced malignancies. Infusion of cytomegalovirus (CMV)-specific CTL clones generated from HLA-matched bone marrow donors resulted in the reconstitution of protective T-cell immunity against CMV (Riddell et al. 1992). In bone marrow recipients, similar results were obtained for restoration of immunity against Epstein-Barr virus (EBV) by adoptive transfer of EBV-specific T-cells generated from the donor (Rooney et al. 1998).

Although transfer of T-cells is a passive transfer of immunity, this immunotherapeutic strategy can potentially activate the endogenous immune system. The transferred T-cells can induce a cascade of cellular interactions leading to the initiation of an endogenous immune response. It has recently been shown that the dynamic interaction of CTL, T helper cells and antigen-presenting DC is required for the initiation of an immune response essential for antigen-specific tumor

rejection. Dendritic cells take up antigens released from dead tumor cells and subsequently process and present antigenic peptides in the context of HLA class I and class II molecules to CD8⁺ and CD4⁺ T-cells respectively. In this scenario, antigen-specific CD4⁺ T-cells provide direct and indirect help to CD8⁺ effector T-cells (Ridge et al. 1998, Albert et al. 2001). During this three-cell interaction, CD8⁺ CTLs are not a passive partner, but are able to activate naive CD4⁺ T helper cells (Stuhler et al. 1999). Therefore, adoptively transferred CD4⁺ T-cells may help pre-existing CD8⁺ CTLs, and *vice versa*.

Attempts to treat HER-2/neu-overexpressing tumors by adoptive transfer of HER-2/neu reactive T-cells have been limited due to the difficulty of generating and expanding autologous CTL and T-helper cells directed against the HER-2/neu antigen. As discussed, *ex vivo* expansion of HER-2/neu-specific T-cells may be facilitated by increasing starting numbers of cultured cells by active immunization. Recently, investigators have developed a protocol using DC retrovirally transduced with the HER-2/neu gene for specific stimulation of autologous peripheral blood lymphocytes (Bernhard et al. 2000, Meyer zum Buschenfelde et al. 2000). HER-2/neu-transduced DC were capable of presenting multiple epitopes and subsequently induced HER-2/neu-specific cytotoxic and helper T-cells in individual donors (Meyer zum Buschenfelde et al. 2001). Both HER-2/neu reactive CD8⁺ CTL and CD4⁺ T-helper cells could be elicited and cloned from a patient with advanced HER-2/neu-overexpressing breast cancer. One of the advantages of using genetically modified DC as APC is the simultaneous stimulation of CTL and T-helper cells recognizing the same antigen in context with different HLA molecules. Clinical trials are poised to examine the therapeutic effect of the infusion of HER-2/neu-specific T-cell clones in order to define the immunological and clinical effect of certain T-cell populations following transfer. In the future, adoptive transfer of HER-2/neu-specific T-cell lines might be preferred to T-cell clones that recognize a single epitope (Knutson & Disis 2001).

Currently, one of the most widely used methods for generating CTL *in vitro* is the use of peptide-pulsed DC as APC. One advantage of using peptide-pulsed DC is the ability to isolate T-cells recognizing subdominant epitopes to which T-cells may not be elicited by stimulation with genetically modified DC. This culture method, however, is often not successful in generating antigen-specific T-cells, due to the low frequency of peptide-specific T-cells even after repetitive IVS. Moreover, this method often promotes the growth of peptide-specific T-cells with low affinity TCR that are unable to lyse tumor cells. The disadvantages of this method can be circumvented by sorting low-frequency antigen-specific T-cells with a high affinity TCR using HLA/peptide fluorescent tetramers (Altman et al. 1996). Using tetramer-guided sorting, high avidity melanoma-reactive CTLs have been isolated from heterogeneous populations that efficiently lyse tumor cells (Yee et al. 1999, Dutoit et al. 2001). However,

other groups have shown that structural avidity does not necessarily translate into high functional avidity (Bullock *et al.* 2001). Using tetramer-guided sorting, it is possible to isolate tumor-reactive HLA-A2-restricted CTLs against the HER-2/neu-derived peptide, p369–377, both from healthy donors and from patients with breast cancer (Schmidt *et al.* 2001).

HER-2/neu-specific T-cells can be expanded *ex vivo* after active immunization

The optimal conditions for culturing T-cells are not yet defined. Exogenous addition of the cytokines IL-7 and IL-15 to the T-cell culture might prevent activation-induced cell death that occurs upon antigen stimulation in the presence of IL-2 (Lynch & Miller 1994, Marks-Konczalik *et al.* 2000). In addition, IL-12 may prove to be a useful cytokine for T-cell expansion. Studies have evaluated the use of IL-12 for the polyclonal proliferation of both influenza-specific CTL and HER-2/neu-specific CD4⁺ T-cells (K L Knutson & M L Disis, unpublished observations). T-cells were expanded from the blood of patients immunized with a vaccine that contained a helper epitope, p776–790, derived from the ICD of HER-2/neu. This particular epitope has been shown to be broadly restricted in response (Sotiriadou *et al.* 2001). While immunity to p776–790 could be readily measured in short-term cultures, cell line generation by multiple IVS with peptide and IL-2 as the only added cytokine resulted in loss of activity. The inclusion of IL-12, along with IL-2, restored antigen-specific proliferation in a dose-dependent fashion. The resulting p776–790-specific T-cells responded readily to antigen by proliferating and producing type I cytokines (IFN γ and tumor necrosis factor α). The increased proliferative response of the cultures was due, in part, to an increase in the number of HER-2/neu-specific T-cells. IL-12 inclusion in the culture media also resulted in the decrease of non-specific cellular proliferation. These results suggest that IL-12 is an important cytokine for *ex vivo* recovery and maintenance of antigen-specific CD4 T lymphocytes that would otherwise be lost by using IL-2 alone in combination with antigen. The further development of defined culture conditions for growing T-cells with a defined phenotype is warranted.

The ability to grow and expand antigen-specific T-cells that are functionally active *in vitro* is a prerequisite, but not a guarantee, for the clinical success of adoptive T-cell transfer. *In vitro* assays for documenting the efficacy of tumor-reactive T-cells by measuring cytotoxicity or cytokine release, are not able to predict the *in vivo* activity of these T-cells following transfer. Multiple factors playing a role during the interaction of effector and tumor cells might prevent T-cells, which efficiently kill tumor cells *in vitro*, from eradicating tumor cells *in vivo* (Dudley *et al.* 2001). On the other hand, T-cells that display low cytolytic function *in vitro* might be good 'killers' *in vivo* (Lynch & Miller 1994). One novel method of improving HER-2/neu-specific CTL

function *in vivo* after infusion of cells is the use of concurrent trastuzumab therapy with adoptive immunotherapy.

Synergistic activity of HER-2/neu-specific CTL cells and a HER-2/neu-specific monoclonal antibody, trastuzumab

Lytic activity of separated HER-2/neu-specific CTL has been low, in part, because HER-2/neu is a self-antigen and T-cells may display a low affinity to T-cell receptors due to induction of tolerance (Fisk *et al.* 1995, Linehan *et al.* 1995, Meyer zum Buschenfelde *et al.* 2000). On this basis, the lytic potential of HER-2/neu-specific CTL could, theoretically, be improved by further increasing the number of HLA class I-bound peptides on tumor cells with the help of trastuzumab, an inhibitory antibody against HER-2/neu. Upon binding of trastuzumab, the HER-2/neu receptor is internalized and degraded, subsequently inhibiting HER-2/neu-mediated signal transduction and tumor cell growth (Carter *et al.* 1992, Hurwitz *et al.* 1995). As antibody-induced degradation of HER-2/neu is likely to be accompanied with increased numbers of HER-2/neu peptides presented with HLA molecules, investigators questioned whether trastuzumab-treated tumor cells were more susceptible to CTL-mediated lysis. Indeed, HER-2/neu reactive CTL clones lyse class I-matched, HER-2/neu-overexpressing tumor cells more efficiently after treatment with trastuzumab (Meyer zum Buschenfelde *et al.* 2002). The potentially synergistic activity of HER-2/neu-specific antibody and CTL encourages the development of HER-2/neu targeted immunotherapy using a combination of inhibitory antibodies and infused CTL for the treatment of patients with HER-2/neu-overexpressing tumors. Planned studies will focus on the toxicity and efficacy of adoptively transferred HER-2/neu-specific CTL with and without trastuzumab in patients with HER-2/neu-overexpressing breast cancer.

Conclusion

Early results of clinical trials actively immunizing cancer patients against HER-2/neu demonstrate that immunity can be generated and that immune responses persist over a period of time. Current vaccine trials have focused solely on the use of epitope- or peptide-based vaccines, largely due to the observation that peptide vaccine strategies could circumvent neu-specific tolerance in rodent models. The next generation of vaccine approaches will include protein-based vaccines, HER-2/neu antigen preparations loaded onto DC, and nucleic acid based formulations. Studies in rodent models exploring these strategies at a pre-clinical level are promising. Expansion of HER-2/neu-specific T-cell *ex vivo* following active immunization or *in vitro* culture with HER-2/neu-expressing DC may be a therapeutic option for treating advanced stage HER-2/neu-overexpressing tumors.

Acknowledgements

This work is supported by grants from the Research Council of Germany (SFB 456), the Wilhelm Sander-Stiftung, and the Deutsche Krebshilfe to H B, by NIH training grant T32 (HL07093) to L S, by a fellowship from the Department of Defense Breast Cancer Program to K L K and by grants from the NIH, NCI (R01 CA75163 and CA85374, and K24 CA 85218) as well as funding from the Cancer Research Treatment Foundation to M L D. We thank Ms Chalie Livingston for assistance in manuscript preparation.

References

- Albert ML, Jegathesan M & Darnell RB 2001 Dendritic cell maturation is required for the cross-tolerization of CD8⁺ T-cells. *Nature Immunology* 2 1010–1017.
- Allred D, Clark G, Molina R, Tandon A, Schnitt S, Gilchrist K, Osborne C, Tormey D & McGuire W 1992 Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. *Human Pathology* 23 974–979.
- Allred DC, Mohsin SK & Fuqua SA 2001 Histological and biological evolution of human premalignant breast disease. *Endocrine-Related Cancer* 8 47–61.
- Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ & Davis MM 1996 Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274 94–96.
- Bachelez H, Flageul B, Degos L, Boumsell L & Bensussan A 1992 TCR gamma delta bearing T lymphocytes infiltrating human primary cutaneous melanomas. *Journal of Investigative Dermatology* 98 369–374.
- Bernhard H, Lohmann M, Batten WY, Metzger J, Lohr HF, Peschel C, zum Buschenfelde KM & Rose-John S 2000 The gp130-stimulating designer cytokine hyper-IL-6 promotes the expansion of human hematopoietic progenitor cells capable to differentiate into functional dendritic cells. *Experimental Hematology* 28 365–372.
- Boismenu R & Havran WL 1998 Gamma delta T-cells in host defense and epithelial cell biology. *Clinical Immunology and Immunopathology* 8 121–133.
- Brossart P, Stuhler G, Flad T, Stevanovic S, Rammensee HG, Kanz L & Brugger W 1998 Her-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by *in vitro* induced specific cytotoxic T lymphocytes. *Cancer Research* 58 732–736.
- Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L & Brugger W 2000 Induction of cytotoxic T-lymphocyte responses *in vivo* after vaccinations with peptide-pulsed dendritic cells. *Blood* 96 3102–3108.
- Bullock TN, Mullins DW, Colella TA & Engelhard VH 2001 Manipulation of avidity to improve effectiveness of adoptively transferred CD8(+) T-cells for melanoma immunotherapy in human MHC class I-transgenic mice. *Journal of Immunology* 167 5824–5831.
- Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME & Shepard HM 1992 Humanization of an anti-p185HER2 antibody for human cancer therapy. *PNAS* 89 4285–4289.
- Cheever MA & Chen W 1997 Therapy with cultured T-cells: principles revisited. *Immunological Reviews* 157 177–194.
- Choudhary A, Davodeau F, Moreau A, Peyrat MA, Bonneville M & Jotereau F 1995 Selective lysis of autologous tumor cells by recurrent gamma delta tumor-infiltrating lymphocytes from renal carcinoma. *Journal of Immunology* 154 3932–3940.
- Craft N, Shostak Y, Carey M & Sawyers CL 1999 A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nature Medicine* 5 280–285.
- Dakappagari NK, Douglas DB, Triozzi PL, Stevens VC & Kaumaya PT 2000 Prevention of mammary tumors with a chimeric HER-2 B-cell epitope peptide vaccine. *Cancer Research* 60 3782–3789.
- Disis ML & Cheever MA 1998 HER-2/neu oncogenic protein: issues in vaccine development. *Critical Review of Immunology* 18 37–45.
- Disis ML, Smith JW, Murphy AE, Chen W & Cheever MA 1994 *In vitro* generation of human cytotoxic T-cells specific for peptides derived from the HER-2/neu proto-oncogene protein. *Cancer Research* 54 1071–1076.
- Disis ML, Bernhard H, Shiota FM, Hand SL, Gralow JR, Huseby ES, Gillis S & Cheever MA 1996a Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood* 88 202–210.
- Disis ML, Gralow JR, Bernhard H, Hand SL, Rubin WD & Cheever MA 1996b Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, an oncogenic self-protein. *Journal of Immunology* 156 3151–3158.
- Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S & Cheever MA 1997 High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. *Journal of Clinical Oncology* 15 3363–3367.
- Disis ML, Knutson KL, Schiffman K, Rinn K & McNeel DG 2000 Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. *Breast Cancer Research Treatment* 62 245–252.
- Disis ML, Gooley T, Rinn K, Davis D, Piepkorn M, Cheever MA, Knutson K & Schiffman K 2002a Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. *Journal of Clinical Oncology* In Press.
- Disis M, Rinn K, Knutson K, Davis D, Caron D, dela Rosa C & Schiffman K 2002b Flt2 ligand as a vaccine adjuvant in association with HER-2/neu peptide based vaccines in patients with HER-2/neu overexpressing cancers. *Blood* In Press.
- Dudley ME, Wunderlich J, Nishimura MI, Yu D, Yang JC, Topalian SL, Schwartzentruber DJ, Hwu P, Marincola FM, Sherry R, Leitman SF & Rosenberg SA. 2001 Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *Journal of Immunotherapy* 24 363–373.
- Dutoit V, Rubio-Godoy V, Dietrich PY, Quiqueres AL, Schnuriger V, Rimoldi D, Lienard D, Speiser D, Guillaume P, Batard P, Cerottini JC, Romero P & Valmori D 2001 Heterogeneous T-cell response to MAGE-A10(254–262): high avidity-specific cytolytic T lymphocytes show superior antitumor activity. *Cancer Research* 61 5850–5856.
- Fisk B, Blevins TL, Wharton JT & Ioannides CG 1995 Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T

- lymphocyte lines. *Journal of Experimental Medicine* **181** 2109–2117.
- Fisk B, Savary C, Hudson JM, O'Brian CA, Murray JL, Wharton JT & Ioannides CG 1996 Changes in a HER-2/neu peptide upregulating HLA-A2 expression affect both conformational epitopes and CTL recognition: implication for optimization of antigen presentation and tumor-specific CTL induction. *Journal of Immunotherapy* **18** 197–209.
- Hayday A & Geng L 1997 Gamma delta cells regulate autoimmunity. *Current Opinion in Immunology* **9** 884–889.
- Hurwitz E, Stancovski I, Sela M & Yarden Y 1995 Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. *PNAS* **92** 3353–3357.
- Ioannides CG, Fisk B, Fan D, Biddison WE, Wharton JT & O'Brian CA 1993 Cytotoxic T-cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. *Cellular Immunology* **151** 225–234.
- Kawashima I, Hudson SJ, Tsai V, Southwood S, Takesako K, Appella E, Sette A & Celis E 1998 The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. *Human Immunology* **59** 1–14.
- Keogh E, Fikes J, Southwood S, Celis E, Chesnut R & Sette A 2001 Identification of new epitopes from four different tumor-associated antigens: recognition of naturally processed epitopes correlates with HLA-A*0201-binding affinity. *Journal of Immunology* **167** 787–796.
- Knutson KL & Disis ML 2001 Expansion of HER-2/neu specific T-cells *ex vivo* following immunization with a HER-2/neu peptide-based vaccine. *Clinical Breast Cancer* **2** 73–79.
- Knutson KL, Schiffman K, & Disis ML 2001 Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *Journal of Clinical Investigation* **107** 477–484.
- Knutson K, Schiffman K, Cheever MA & Disis M 2002 Immunization of cancer patients with a HER-2/neu HLA-A2 peptide, p369–377, results in short-lived peptide specific immunity. *Clinical Cancer Research* In Press.
- Kono K, Rongcun Y, Charo J, Ichihara F, Celis E, Sette A, Appella E, Sekikawa T, Matsumoto Y & Kiessling R 1998 Identification of HER2/neu-derived peptide epitopes recognized by gastric cancer-specific cytotoxic T lymphocytes. *International Journal of Cancer* **78** 202–208.
- Lehmann PV, Forsthuber T, Miller A & Sercarz EE 1992 Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* **358** 155–157.
- Linehan DC, Goedegebuure PS, Peoples GE, Rogers SO & Eberlein TJ 1995 Tumor-specific and HLA-A2-restricted cytotoxicity by tumor-associated lymphocytes in human metastatic breast cancer. *Journal of Immunology* **155** 4486–4491.
- Lynch DH 1998 Induction of dendritic cells (DC) by Flt3 ligand (FL) promotes the generation of tumor-specific immune responses *in vivo*. *Critical Reviews in Immunology* **18** 99–107.
- Lynch DH & Miller RE 1994 Interleukin 7 promotes long-term *in vitro* growth of antitumor cytotoxic T lymphocytes with immunotherapeutic efficacy *in vivo*. *Journal of Experimental Medicine* **179** 31–42.
- Mach N, Gillesen S, Wilson SB, Sheehan C, Mihm M & Dranoff G 2000 Differences in dendritic cells stimulated *in vivo* by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Research* **60** 3239–3246.
- McNeel DG, Nguyen LD, Storer BE, Vessella R, Lange PH & Disis ML 2000 Antibody immunity to prostate cancer-associated antigens can be detected in the serum of patients with prostate cancer. *Journal of Urology* **164** 1825–1829.
- Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K & McKenna HJ 1996 Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *Journal of Experimental Medicine* **184** 1953–1962.
- Maraskovsky E, Daro E, Roux E, Teepe M, Maliszewski CR, Hoek J, Caron D, Lebsack ME & McKenna HJ 2000 *In vivo* generation of human dendritic cell subsets by Flt3 ligand. *Blood* **96** 878–884.
- Marks-Konczalik J, Dubois S, Losi JM, Sabzevari H, Yamada N, Feigenbaum L, Waldmann TA & Tagaya Y 2000 IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *PNAS* **97** 11445–11450.
- Meyer zum Buschenfelde C, Nicklisch N, Rose-John S, Peschel C & Bernhard H 2000 Generation of tumor-reactive CTL against the tumor-associated antigen HER2 using retrovirally transduced dendritic cells derived from CD34⁺ hemopoietic progenitor cells. *Journal of Immunology* **165** 4133–4140.
- Meyer zum Buschenfelde CM, Metzger J, Hermann C, Nicklisch N, Peschel C & Bernhard H 2001 The generation of both T killer and Th cell clones specific for the tumor-associated antigen HER2 using retrovirally transduced dendritic cells. *Journal of Immunology* **167** 1712–1719.
- Meyer zum Buschenfelde C, Hermann C, Schmidt B, Peschel C & Bernhard H 2002 Anti-HER2 monoclonal antibody trastuzumab enhances cytolytic activity of class I restricted HER2-specific T lymphocytes against HER2-overexpressing tumor cells. *Cancer Research* In Press.
- Nanda NK & Sercarz EE 1995 Induction of anti-self-immunity to cure cancer. *Cell* **82** 13–17.
- Olayioye MA, Neve RM, Lane HA & Hynes NE 2000 The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO Journal* **19** 3159–3167.
- Peiper M, Goedegebuure PS, Izbicki JR & Eberlein TJ 1999 Pancreatic cancer associated ascites-derived CTL recognize a nine-amino-acid peptide GP2 derived from HER2/neu. *AntiCancer Research* **19** 2471–2475.
- Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I & Eberlein TJ 1995 Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. *PNAS* **92** 432–436.
- Pulendran B, Smith JL, Jenkins M, Schoenborn M, Maraskovsky E & Maliszewski CR 1998 Prevention of peripheral tolerance by a dendritic cell growth factor: flt3 ligand as an adjuvant. *Journal of Experimental Medicine* **188** 2075–2082.
- Riddell S, Watanabe K, Goodrich J, Li C, Agha M & Greenberg P 1992 Restoration of viral immunity in immunocompromised humans by the adoptive transfer of T-cell clones. *Science* **257** 238–241.
- Ridge JP, Di Rosa F & Matzinger P 1998 A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and a T-killer cell. *Nature* **393** 474–478.
- Rongcun Y, Salazar-Onfray F, Charo J, Malmberg KJ, Evrin K, Maes H, Kono K, Hising C, Petersson M, Larsson O, Lan L, Appella E, Sette A, Celis E & Kiessling R 1999 Identification of new HER2/neu-derived peptide epitopes that can elicit specific

- CTL against autologous and allogeneic carcinomas and melanomas. *Journal of Immunology* 163 1037–1044.
- Rooney CM, Smith CA, Ng CY, Loftin SK, Sixbey JW, Gan Y, Srivastava DK, Bowman LC, Krance RA, Brenner MK & Heslop HE 1998 Infusion of cytotoxic T-cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 92 1549–1555.
- Samanta A, LeVea CM, Dougall WC, Qian X & Greene MI 1994 Ligand and p185c-neu density govern receptor interactions and tyrosine kinase activation. *PNAS* 91 1711–1715.
- Schmidt B, Busch B, Schulz E, Hermann C, Wagner H, Peschel C & Bernhard H 2001 Generation and expansion of HER2-reactive T-cell clones for adoptive immunotherapy. *Onkologie* 24 S6 (Abstract).
- Sercarz EE 2000 Driver clones and determinant spreading. *Journal of Autoimmunity* 14 275–277.
- Sercarz EE, Lehmann PV, Ametani A, Benichou G, Miller A & Moudgil K 1993 Dominance and crypticity of T-cell antigenic determinants. *Annual Review of Immunology* 11 729–766.
- el-Shami K, Tirosh B, Bar-Haim E, Carmon L, Vadai E, Fridkin M, Feldman M & Eisenbach L 1999 MHC class I-restricted epitope spreading in the context of tumor rejection following vaccination with a single immunodominant CTL epitope. *European Journal of Immunology* 29 3295–3301.
- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A & McGuire WL 1987 Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235 177–182.
- Smorlesi A, Shiota F & Disis ML 1999 Flt3-ligand (Flt3l), as a vaccine adjuvant, results in the generation of antigen-specific IFN γ producing T-cells after immunization. *Proceedings of the American Association for Cancer Research* 40 313–313.
- Somasundaram R, Robbins P, Moonka D, Loh E, Marincola F, Patel A, Guerry D & Herlyn D 2000 CD4(+), HLA class I-restricted, cytolytic T-lymphocyte clone against primary malignant melanoma cells. *International Journal of Cancer* 85 253–259.
- Sotiriadou R, Perez SA, Gritzapis AD, Sotiropoulou PA, Echner H, Heinzel S, Mamalaki A, Pawelec G, Voelter W, Baxevas CN & Papamichail M 2001 Peptide HER2(776–788) represents a naturally processed broad MHC class II-restricted T-cell epitope. *British Journal of Cancer* 85 1527–1534.
- Stark A, Hulka BS, Joens S, Novotny D, Thor AD, Wold LE, Schell MJ, Melton LJ 3rd, Liu ET & Conway K 2000 HER-2/neu amplification in benign breast disease and the risk of subsequent breast cancer. *Journal of Clinical Oncology* 18 267–274.
- Stuhler G, Zobywalski A, Grunebach F, Brossart P, Reichardt VL, Barth H, Stevanovic S, Brugger W, Kanz L & Schlossman SF 1999 Immune regulatory loops determine productive interactions within human T lymphocyte-dendritic cell clusters. *PNAS* 96 1532–1535.
- Vanderlugt CJ & Miller SD 1996 Epitope spreading. *Current Opinion in Immunology* 8 831–836.
- van der Veen RC, Dietlin TA, Pen L & Gray JD 1999 Nitric oxide inhibits the proliferation of T-helper 1 and 2 lymphocytes without reduction in cytokine secretion. *Cellular Immunology* 193 194–201.
- Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S & Stewart SJ 2001 First-line herceptin((r)) monotherapy in metastatic breast cancer. *Oncology* 61 (Suppl S2) 37–42.
- de Vries JE & Spits H 1984 Cloned human cytotoxic T lymphocyte (CTL) lines reactive with autologous melanoma cells. I. *In vitro* generation, isolation, and analysis to phenotype and specificity. *Journal of Immunology* 132 510–519.
- Ward RL, Hawkins N, Coomber D & Disis ML 1999 Antibody immunity to the HER-2/neu oncogenic protein in patients with colorectal cancer. *Human Immunology* 60 510–515.
- Watanabe N, Hizuta A, Tanaka N & Orita K 1995 Localization of T-cell receptor (TCR)-gamma delta + T-cells into human colorectal cancer: flow cytometric analysis of TCR-gamma delta expression in tumour-infiltrating lymphocytes. *Clinical and Experimental Immunology* 102 167–173.
- Yee C, Savage PA, Lee PP, Davis MM & Greenberg PD 1999 Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *Journal of Immunology* 162 2227–2234.
- Yip YL, Smith G, Koch J, Dubel S & Ward RL 2001 Identification of epitope regions recognized by tumor inhibitory and stimulatory anti-ErbB-2 monoclonal antibodies: implications for vaccine design. *Journal of Immunology* 166 5271–5278.
- Yoshino I, Goedegebuure PS, Peoples GE, Parikh AS, DiMaio JM, Lysterly HK, Gazdar AF & Eberlein TJ 1994 HER2/neu-derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. *Cancer Research* 54 3387–3390.
- Yu S, He W, Chen J, Zhang F & Ba D 1999 Expansion and immunological study of human tumor infiltrating gamma/delta T lymphocytes *in vitro*. *International Archives of Allergy and Immunology* 119 31–37.
- Zaks TZ & Rosenberg SA 1998 Immunization with a peptide epitope (p369–377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu⁺ tumors. *Cancer Research* 58 4902–4908.
- Zhao X, Wei YQ, Kariya Y, Teshigawara K & Uchida A 1995 Accumulation of gamma/delta T-cells in human dysgerminoma and seminoma: roles in autologous tumor killing and granuloma formation. *Immunological Investigations* 24 607–618.